The intimate interactions of food isoflavones with estrogen receptors, corresponding receptor-driven activities, and their potential health consequences

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A thesis submitted in partial fulfilment of the requirements for the

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by Hui Ye

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Abstract

Estrogen receptors (ERs), are members of the super family of ligand-regulated nuclear transcription factors, mediate the action of estrogens, including its primary endogenous ligand 17β-estradiol (E2). ERs have two isoforms, namely estrogen receptor α (ERα) and estrogen receptor β (ERβ). One of the functional domains of ER—ligand binding domain (LBD) has two binding sites; the ligand binding cleft (LBC) directly interacts with the ligands, and the second binding cleft—action function 2 (AF-2) hosts a coactivator or corepressor protein, which up- or down-regulate ER-mediated transcription. ERs are promiscuous, this allows many xenobiotic compounds that have structural similarities with E2 can bind at the LBC. These compounds are xenoestrogens (e.g., genistein in soy foods, bisphenol-A in plastics). Despite the crucial role of ERs in human health, little is known about the details of the communication between their two binding sites and the precision of interactions between the LBC with enormous number of xenoestrogens presenting in environment and foods. These complex interactions were studied in the research described in this thesis using molecular modelling system (Schrödinger).

The first part of this thesis investigates the architectural communication between the two binding sites (LBC and AF-2); this provides a basis for understanding the different levels of ER's biological activities. In this work, Schrödinger (a molecule computational platform) is used to study the interactions between ligands (i.e., in this thesis, flavonoids) and the LBC; this indicates the potential effects of structural features of ligands on their binding energies and binding affinities with ERs, thus differentiating ligands' ERs-driven bioactivities. The

second part of this thesis describes studies on structure-activity relationships for dietary xenoestrogens-isoflavones with ERα using a gene reporter bioassay (MELN assay). The third part of the thesis uses a cell proliferation study (Caco-2 cell line) to study the structure-activity relationships of selected isoflavones with ERβ. In this part, gallic acid, an inhibitor of UDP-glucose dehydrogenase, is used to interfere with the conjugation of isoflavones; this investigates the effect of intestinal phase II metabolism on isoflavone's bioactivity (i.e., ER-driven activity). The last part of the thesis uses an *in vitro* gut fermentation system to investigate the effects of selected isoflavones gut bacterial populations, and uses a Caco-2 monolayer system to study the absorption and metabolism of the isoflavones.

The studies show that, the communication between the two binding sites (LBC and AF-2) via sharing helix components is triggered by ligands docked at the LBC. Ligands with different structural properties can initiate different degrees of conformational changes in the LBC, resulting in different knock-on effects on AF-2, this facilities different amino acid residue orientations change in AF-2, which would form correspondingly different noncovalent interactions with the regulatory protein. This, in turn, likely determines the bioactivity of ERs. In addition, the precision of interactions between ligands (i.e., flavonoids) and LBC indicates the polar substitution arrangements (i.e., hydroxyl groups) might affect the binding energy and binding affinity, and thus, likely results in different ER-mediated bioactivities. This finding is supported by increased ligand output from isoflavones-exposed MELN cells and increase proliferation of ERβ-expressing Caco-2 cells exposed to isoflavones. In addition, the UDP-glucose 6-dehydrogenase inhibitor, gallic acid increases the proliferative ERβ-driven effect of isoflavones on Caco-2 cells, because it prevented phase II metabolism of isoflavones. In the *in vitro* gut fermentation experiments, isoflavones change gut bacterial

populations, and these changes are likely positive in a human health setting. Finally, *in vivo* cell culture experiment shows isoflavones are taken up by a Caco-2 cell monolayer model system which mimics the gut mucosa; this indicates that isoflavones might be absorbed and metabolised by gut mucosa.

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Name: Professor Ian Shaw Signature:  Date: 1st November, 2018

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Abbreviations

6-OH-DAID	6-hydroxydaidzein
8-OH-DAID	8-hydroxydaidzein
3′-OH-DAID	3′-hydroxydaidzein
6-OH-GEN	6-hydroxygenistein
8-OH-GEN	8-hydroxygenistein
3′-OH-GEN	3′-hydroxygenistein
17β-HSD	17β-Hydroxysteroid dihydrogen
AIDS	Acquired Immune Deficiency Syndrome
AF-1	Activation function 1
AF-2	Activation function 2
AP	Apical
AQs	Anthraquinone derivatives
Bcl-2	B-cell lymphocyte/leukemia-2
BL	Basolateral
BPA	Bisphenol A
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CAULX	Chemical activated luciferase gene expression
cDNA	Complementary DNA

CHD	Coronary heart disease
CNS	Central nervous system
CPK	Corey-Pauling-Koltun
CYP450	Cytochrome P450
DAID	Daidzein
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DPN	2,3-Bis (4-hydroxyphenyl) propionitrile
DPPH	1,1-Diphenyl-2-picrylhydrazyl
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDCs	Endocrine disrupting chemicals
EDTA	Ethylenediaminetetraacetic Acid
EE2	17α-Ethinylestradiol
ER1	Estrogen receptor 1
ERE	Estrogen response element
ERs	Estrogen receptors
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
E-Screen	Estrogenicity screen

Et	Ethyl
ETP	Ethoxytriphenylethene
F/B	Firmicutes/Bacteriodetes
FBS	Fetal bovine serum
FOR	Formononetin
GA	Gallic acid
GEN	Genistein
H	Helix
H-bonds	Hydrogen bonds
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HER	Hydrophobic enclosure
HIV-1	Human immunodeficiency virus 1
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
LBC	Ligand binding cleft
LBD	Ligand binding domain
LDL	Low density lipoprotein
MEM	Minimal essential medium
MELN	MCF-7 cells transfected with ERE-Glob-Luc-SVNeo plasmid
ND	Not detected
NRs	Nuclear receptors

OBHS	Oxabicyclic heptene sulfonate
OHD	Ortho-hydroxydaidzein
OHG	Ortho-hydroxygenistein
ODS	Octadecylsilane
PDB	Protein Data Bank
P_{app}	Permeability coefficient
RAA	Relative agonistic activity
RBA	Relative binding affinity
RIPA	Radioimmunprecipition
RLU	Relative light units
RMSD	Root-mean-square deviation
RRA	Relative recruitment ability
SCFAs	Short chain fatty acids
TEER	Transepithelial electrical resistance
t_R	Retention time
TRs	Transport rates
TR	Transport rate
UDP-glucuronic acid	Uridine diphosphate glucuronic acid
UGDH	UDP-glucose dehydrogenase
US	United States
UV	Ultraviolet

WCA broth	Wilkins chalgren anaerobic broth
WHO	World Health Organization
XO	Xanthine oxidase
XP	Extra precision
YES assay	Yeast estrogenicity screen
λ	Wavelength

Chapter 1: Introduction

1 Chapter 1 – Introduction

1.1 Food functionality

Food is any substance consumed to provide nutritional support for an organism. It is of plant or animal origin, and contains essential nutrients, such as carbohydrates, fats, proteins, vitamins, and minerals.[1] Originally, food functionality was regarded as the ability of such substrates to be ingested and assimilated by an organism's cells to provide energy, maintain life, or stimulate growth.[2] Food functionality is gaining significant importance with more in-depth understanding of the risks and benefits associated with foods-health promotion and disease prevention.[1] An understanding of the possible mechanisms of biological activity of functional food components is important if they are to be developed in a health promotion setting. Interestingly, flavonoids have been extensively studied as a key functional component that is linked to health promotion. China has been regarded as the origin of "medicinal and edible plants".[3] Ginger is a traditional Chinese medicine which has been used to help prevent the common cold; this is likely due to ginger containing low-melt fatty acids, this fatty acid may release energy during evaporation *in vivo*, but more importantly, ginger is rich in curcumin (a flavonoid), which can inhibit the rhinovirus (the most common family of cold viruses).[4] Another example is goji berry, which is recommended for its benefits for eyesight. The key compounds found in goji berry, are lutein and zeaxanthin, both of which have been reported to prevent cataracts and macular degeneration. These compounds may act to protect the eyesight from ultraviolet phototoxicity *via* quenching reactive oxygen species.[5, 6] In Tunisia, *T. gallica* has been used as a traditional treatment for liver disorder, because *Tamarix gallica* contains the flavonoids, 5-hydroxy-3,7, 4'-trimethoxyflavone and 3,5,7-trihydroxy-4'-methoxyflavone, which are known for their heptatonic and stimulant properties.[7]

In addition, some flavonoid-containing foods have been linked to sexual development and reproduction.[8] In ancient China, people fed rams with dry leaves of epicedium during the breeding season.[8, 9] This ancient tradition is in fact supported by a study that showed that icariin, a functional component of epicedium, has testosterone-mimicking properties.[10] On

the other hand, in the United Kingdom, red clover is used to increase the milk yielding capacity of goats, this is due to the high concentration of formononetin (FOR) which is an estrogen mimic can occupy and activate ERs. In addition, tofu (a soybean-based food) is used to increase breast-milk production, and the higher soy consumption in Japan compared with the United States (US) is linked to a lower incident rate for breast cancer in Japan (more than 80% of breast cancer is estrogen positive).[11] Soy isoflavones are estrogen mimics because of their similarity in structure to the natural estrogen 17β-estradiol (E2) which activates estrogen receptors (ERs) and initials estrogenic responses.[12] Indeed, these isoflavones (e.g., genistein (GEN), daidzein (DAID) have been proved to occupy and activate ERs.[13]

1.2 Estrogen receptors and estrogens

1.2.1 Overview

Steroid hormone estrogens influence the growth, differentiation, and functioning of many higher target tissues and cell types, and ERs have two isoforms, namely estrogen receptor α (ERα) and estrogen receptor β (ERβ).[14, 15] These two isoforms of ER have highly similar protein architecture but with different amino acid orientations.[16] The bio-action of ERs is triggered *via* ligand binding at a ligand binding cleft (LBC), and its consequential knock-on effects for facilitating the second binding cleft—activation function 2 (AF-2) to host coactivator or corepressors protein, which up- or down-regulate ER-mediated transcription.[13, 17] ERs' promiscuity allows many xenobiotic compounds that have structural similarity to E2 to bind at the LBC; these xenobiotics are termed xenoestrogens.[13,18]

1.2.2 ERs

ERs, members of the super family of ligand-regulated nuclear transcription factors, mediate the action of estrogens, including the primary endogenous ligand E2. As outlined before (***Section 1.2.1***), ERs have two isoforms, namely ERα and ERβ. ERs have six domains (A–F) including three major functional domains comprising a hypervariable N-terminal domain (containing activation function 1 (AF-1) that contributes to the transactivation function in the

absence of a bound ligand (e.g., E2);[13] a highly conserved central domain responsible for specific DNA binding, dimerization, and nuclear localization; and a C-terminal domain involved in ligand binding and ligand-dependent transactivation function, which is the ligand binding domain (LBD).[13, 19, 20] In addition, the LBD comprises two separate but interacting binding clefts—the LBC and AF-2.[13, 21] The LBC binds a ligand (either agonist or antagonist), which initiates a conformational change that exposes AF-2 to allow its interaction with regulatory proteins (Fig. 1.1).[22] The conformational change initiated by ligand binding activates ERs' dissociation from a heat shock protein (usually Hsp90).[13] Phosphorylation then occurs, which aids receptor dimerization. The dimer then moves into the nucleus and binds to DNA *via* the estrogen response element (ERE) or *via* a protein DNA binding intermediate.[23] Coregulatory protein recruitment then occurs.[13,24] The coregulatory proteins comprise coactivators (promotors of ER activity) and corepressors (suppressors of ER activity). The bound regulatory protein establishes a "triangular relationship" with the ER and the bound ligand that facilitates fine tuning of the estrogenic response.[13, 25]

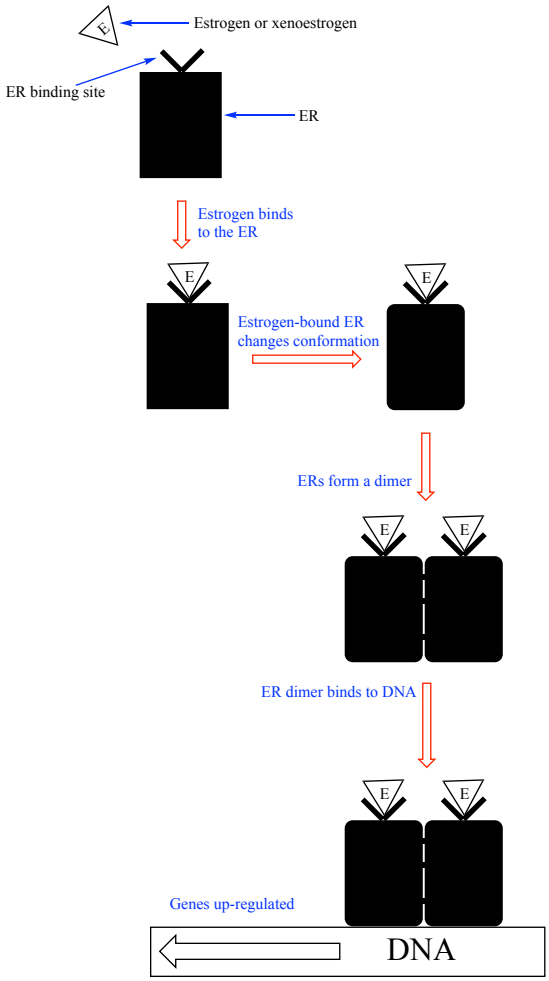


Figure 1.1: Schematic representation of the mechanism of action of estrogens. The estrogen (e.g., E2) binds to the LBC of ER; this initiates a conformational change of ER and facilitating ER dimerization. The ER dimer binds to DNA at a specific binding site and up-regulates specific genes associated with cellular feminisation. Figure from I.C. Shaw Food Safety—The Science of Keeping Food Safe, Wiley-Blackwell. Reproduced with permission of the author.

In the LBD of ERs, helix (H) 3 to H12 (Fig. 1.2) are important in the functional architecture of the LBD; they are folded into three layers—H5/6, H9 and H10—sandwiched between two additional layers comprising H1–4 and H7, H8, H11.[13, 21] The LBC is completely partitioned from the external environment and occupies a relatively large portion of the LBD's hydrophobic core (Fig. 1.2). The LBC comprises segments from H3, H6, H8 and a preceding loop, H11, H12 and the S1/S2 hairpin (Fig. 1.2).[13, 21] The arrangement of these helices creates a 3-dimensional LBC at the narrow end of the LBD with adjacent AF-2. AF-2 is the

hydrophobic cleft formed by the remaining segment of H3 and part of H5, H4 and H12 (Fig. 1.2).[13]

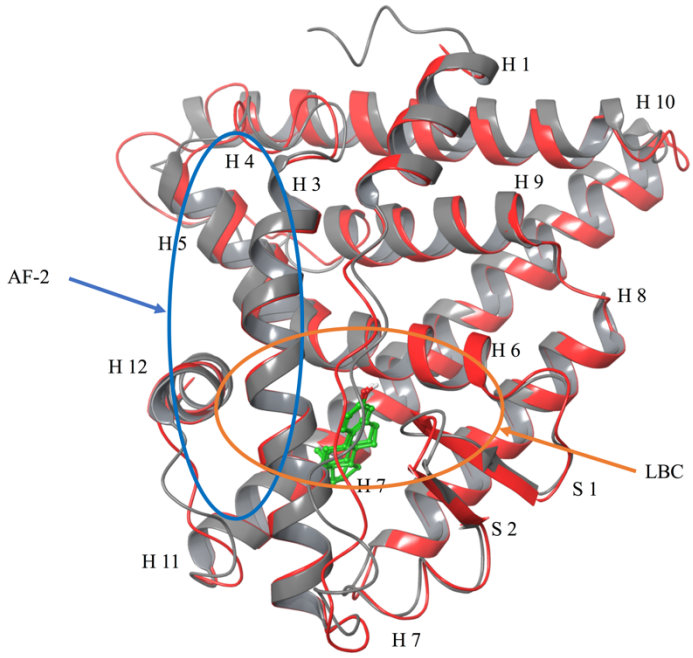


Figure 1.2: Protein structure alignment of ERα (Protein Date Bank (PDB) entry code: 1ERE (grey), ligand: E2) and ERβ (PDB entry code: 3OLS (red), ligand: E2) in agonist conformation. The LBC is shown in an orange ellipse, the AF-2 is shown in a blue ellipse. The ligand is shown in green.

However, the antagonist conformations of ERs are distinctly different from the agonist conformations (Fig. 1.3).[20] Figure 1.3 shows the protein structure alignment of agonist conformation of ERα (PDB entry code: 1ERE, ligand: E2) and antagonist conformation of ERα (PDB entry code:1ERR, ligand: raloxifene); which illustrates H12 was physically obstructed by an antagonist ligand (raloxifene) with a bulky side chain; In addition, the protein structure alignment of agonist conformation of ERβ (PDB entry code:3OLS, ligand: E2) and antagonist conformation of ERβ (PDB entry code: 1QKN, ligand: raloxifene) also shows H12 was pushed away by the antagonist ligand (raloxifene). Both conformational changes of individual ER isomers caused the displacement and dysfunction of AF-2.[20]

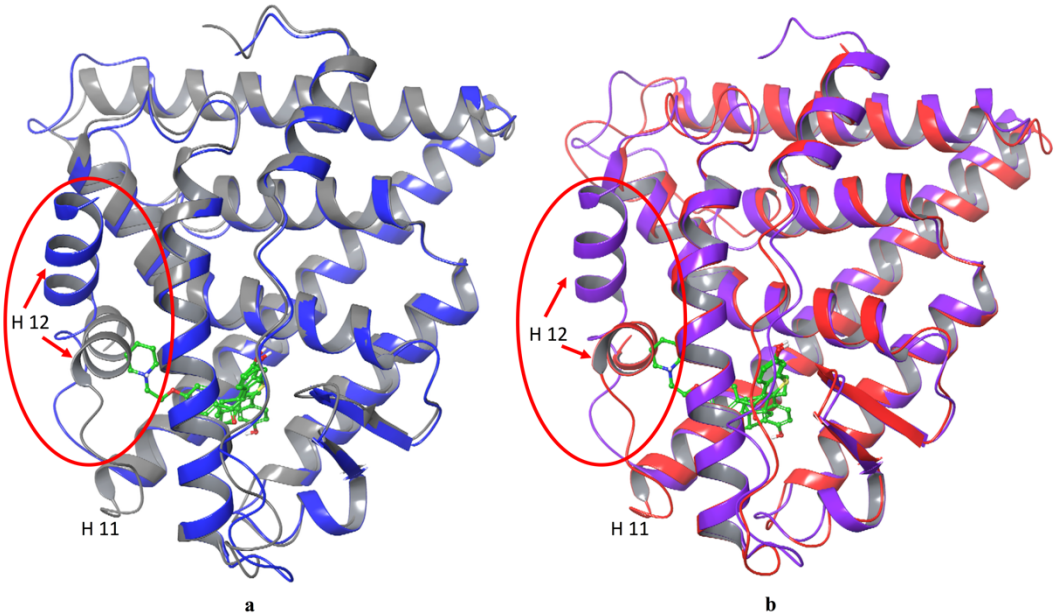


Figure 1.3: a: protein structure alignment of agonist conformation of ERα (Grey, PDB entry code: 1ERE, ligand: E2) and antagonist conformation of ERα (Blue, PDB entry code:1ERR, ligand: raloxifene); b: protein structure alignment of agonist conformation of ERβ (Red, PDB entry code:3OLS, ligand: E2) and antagonist conformation of ERβ (Purple, PDB entry code: 1QKN, ligand: raloxifene). H12s of both agonist and antagonist conformation of ERs are shown in red ellipses. This shows the different orientations of H12 in the agonist and antagonist conformation of ERs. Ligands are shown in green.

1.2.3 Estrogens

Estrogens are important in both female and male.[26] In females, estrogens are responsible for the development of female secondary sex characteristics (e.g. breasts, wider hips, fat distribution) and for regulation of the menstrual cycle (Fig. 1.4).[27] The major sites for the biosynthesis of estrogens are the ovaries and placenta, and to a lesser extent the liver, adrenal cortex, fat cells and mammary glands.[28] The levels of estrogens are increased during pregnancy; this increase is caused by the relative higher production of estrogens in the placenta.[29] In males, estrogens modulate libido, spermatogenesis, and erectile function.[28] The major site of production of estrogens in men is in the testis by the Leydig cells and germ cells affecting normal male gonadal development and spermatogenesis.[30] In addition, estrogens are

produced in the brain which has been linked to energy homeostasis *via* an ER neuronal cluster.[31, 32]

There are three natural estrogens; namely, estrone (E1), E2, and estriol (E3) (Fig. 1.5).[33] E2 is responsible for secondary sex characteristics, puberty onset, and the control of the menstrual and reproductive cycle in women.[34, 35] The level of E2 depends on the age, sex, and stage of the menstrual cycle of an individual.[36-38] The serum E2 concentration in women fluctuates with progression of the menstrual cycle (Fig. 1.4).[36-38] During the follicular phase of the menstrual cycle, E2 increases and peaks as high as 9×10^{-4} mg/L, signalling ovulation (Fig. 1.4).[39] After the release of egg in the ovulation phase, this is the luteal phase with a decrease of E2 and following a plateau phase (Fig. 1.4).[39] However, the concentrations of E2 will fluctuate between the plateau ranges during the luteal phase (Fig. 1.4).[39] The length of the follicular, ovulation, and luteal phase are individually different.[40] Women's serum E2 levels ranges from 3.1×10^{-5} to 4×10^{-4} mg/L; whereas the serum levels of E2 in males pre-pubescent children, and post-menopausal women ranging from 5×10^{-6} to 4.6×10^{-5} mg/L because of the reduction of estrogens.[38]

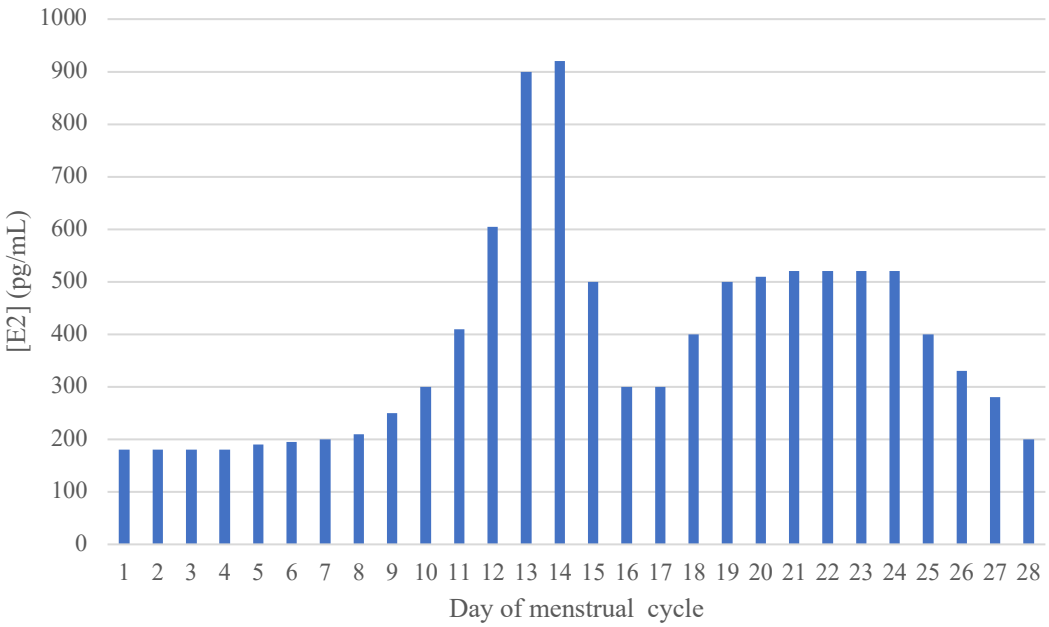


Figure 1.4: Serum levels of circulating E2 in women during the menstrual cycle showing the large fluctuations. Ovulation usually occurs on day 13 or 14. The figure adapted from Shaw IC (2018) Food Safety—The Science of Keeping Food Safe, Wiley-Blackwell, p251, Fig. 9.7. Reproduced with permission of the author.

Estrogenicity describes the physiological activity of a compound compared to E2. E3 has weaker estrogenicity compared with E2, and 'free' E3 (non-protein bound) is almost undetectable in women who are not pregnant;[41] this is due the rapid metabolism of E3 and excretion in urine.[42] However, during pregnancy, E3 is the most biosynthesised estrogen and being increased in the placenta during this time.[43] E3 is also present in high concentrations in bile, meconium, and the corpus luteum.[44] Unlike E1 and E2, E3 is not biosynthesised in the ovaries, instead it is biosynthesised in the liver.[45] This is achieved through the hydroxylation of E1 and E2 by cytochrome P450 (CYP450) 3A4 (Fig. 1.5).[43] In addition, E3 is used as a medication in menopausal hormone therapy to treat menopausal symptoms, such as hot flashes and dyspareunia, but treatment with E3 has been reported to exhibit some side effects including breast tenderness, and endometrial hyperplasia.[44]

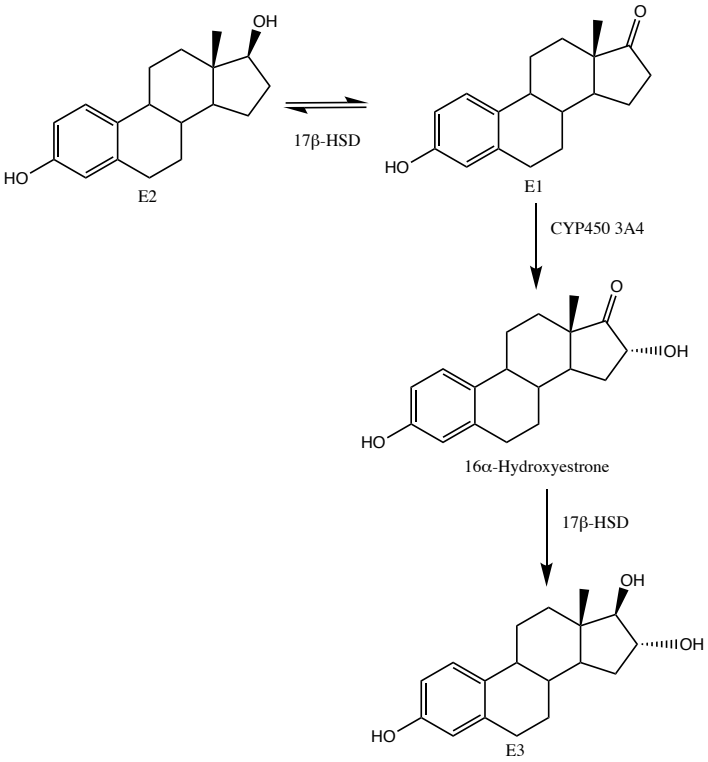


Figure 1.5: Biosynthesis of E3 from E2 via E1 and 16α-hydroxyestrone. 17β-HSD: 17β-hydroxysteroid dehydrogenase.

E1 was the first pure steroid hormone isolated by Adolf Butenandt in 1929 from the urine of pregnant women.[46] Similar to E2, E1 is biosynthesised from cholesterol in the gonads and adipose tissue.[46] Additionally, E1 can be biosynthesised from E2, and E2 can also be reversibly converted into E2 by the enzyme 17β-Hydroxysteroid dehydrogenase (17β-HSD)

(Fig. 1.5), which mainly occur in liver.[15] This reversible conversion allows E1 to be used as clinical treatment for postmenopausal symptoms (e.g., hot flashes, night sweats).[15] However, E1 is now mostly no longer marketed for postmenopausal treatment; this is due to the use of synthetic estrogens (e.g., 17α-Ethinylestradiol (EE2) with better properties including longer activity, higher oral bioavailability, and greater estrogenicity compared with E1.[47]

1.2.4 The metabolism of estrogens

Estrogens circulate in the blood in free or protein-bound forms and would undergo Phase I hepatic metabolism; their irreversible hydroxylation can produce estrogen metabolites at C-2, C-4, or C-16 positions of the steroid ring (e.g., 2-OH-estradiol (Table 5.2), and resulting in different estrogenicities and half-life.[48] In the liver, estrogens are conjugated through glucuronidation or also through sulfonation to allow for biliary excretion.[48] Conjugated estrogens are excreted in bile, urine, and faeces; this has been proved by the injection of radioactively labelled estrogens in women and suggesting biologically significant proportion of estrogens are reabsorbed in the circulation (Fig. 1.6).[48] Hepatically conjugated estrogens excreted in the bile can be deconjugated by bacterial species (e.g., phyla Bacteroidetes and Firmicutes) with β-glucuronidase activity in the gut, leading to the reabsorption into the enterohepatic circulation (Fig. 1.6).[48]

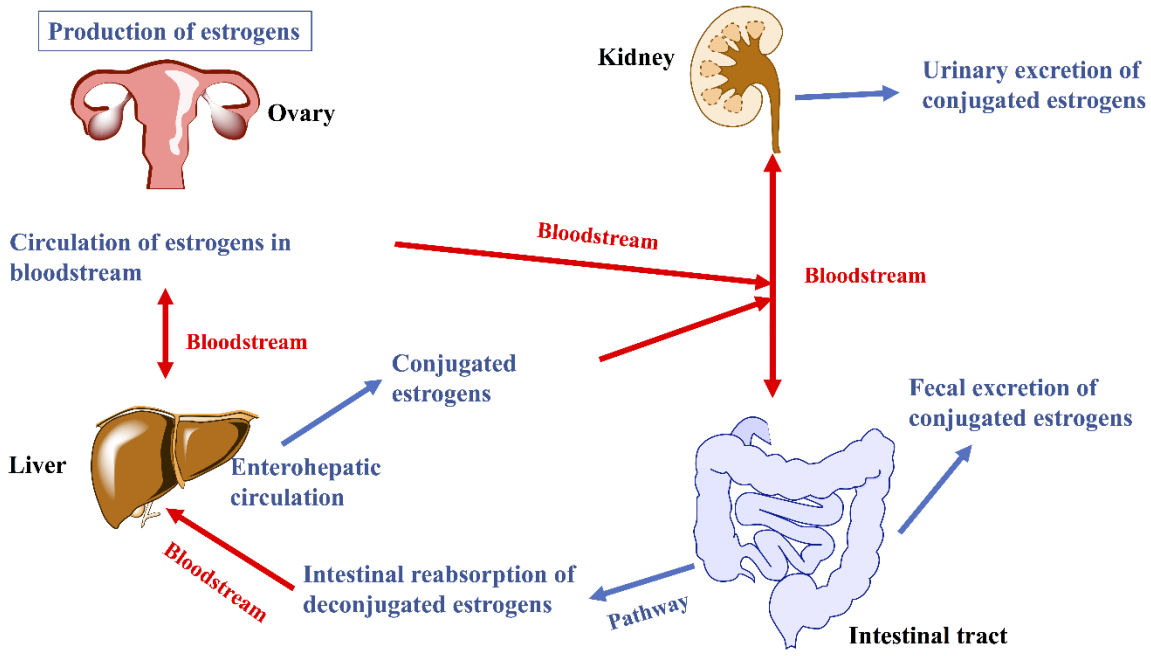


Figure 1.6: The metabolic pathway of estrogens showing that estrogens are biosynthesised in the ovary, estrogens circulate into bloodstream in free or protein-bound forms, then undergo hepatic phase II metabolism. The conjugates are excreted in urine or in bile into the feces. Afterwards, the conjugated estrogens excreted in the bile can be deconjugated by β-glucuronidase from gut bacteria and reabsorbed.

1.2.5 The LBC of ERs

Estrogens mediate their actions through ERs.[49] ERs have specific binding sites for estrogens, the binding sites have conformational binding specificity and binding requirements for ligands (e.g., E2).[50] Figures 1.7 and 1.8 show the LBC of ERα and ERβ, respectively; the overall homology of the LBD between ERα and ERβ is less than 55%, but these two ER isoforms have great homology in their overall architecture (Fig. 1.2). In addition, the LBC, where interacts directly with ligands, of these two isoforms of ERs are remarkable highly homologous and providing similar binding environment and for ligands.[51]

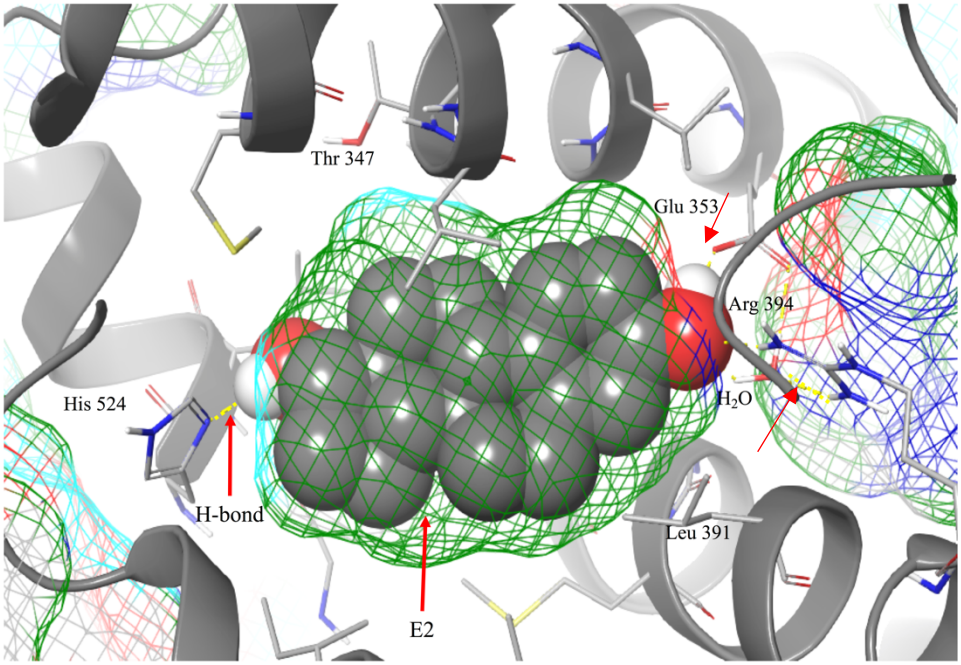


Figure 1.7: X-ray crystal structure of E2 in the binding pocket of ERα showing the H-bonds (---, point out with a red arrow) between the ligand and LBC. Regions of the protein are represented as red for negative, blue for positive, green for nonpolar, cyan for polar, grey for neutral. Amino acid residues interacting with the ligand via H-bonds are shown. All amino acids residues within 3 Å of the ligand are also shown to indicate the chemical nature of the binding pocket.

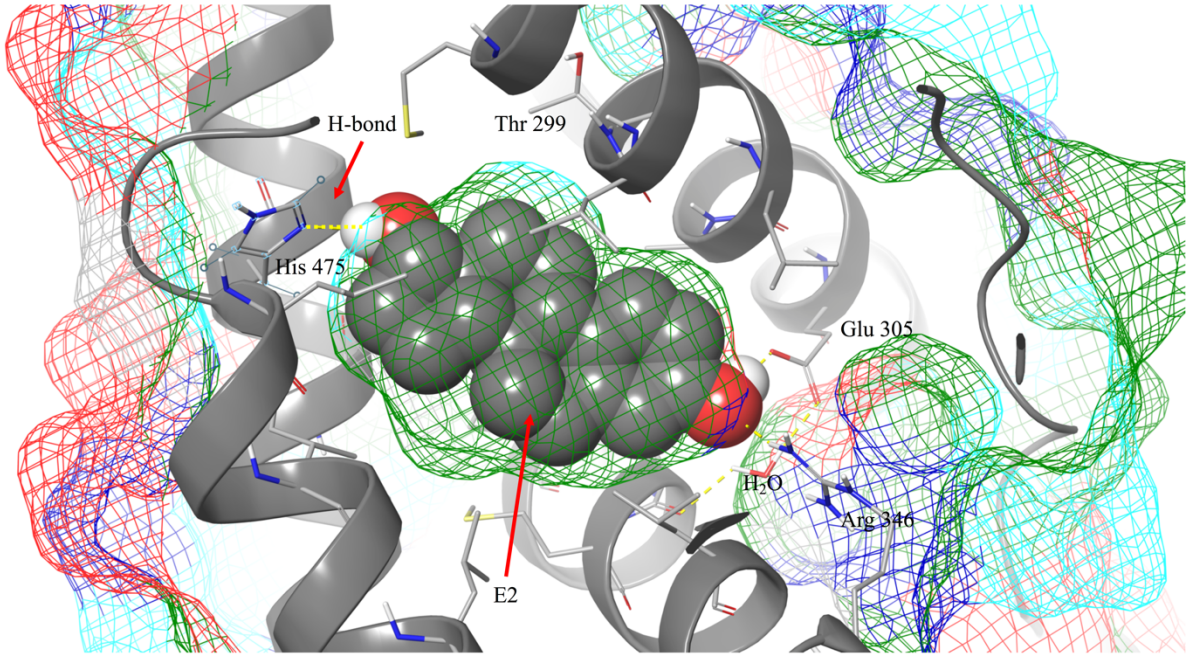


Figure 1.8: X-ray crystal structure of E2 in the binding pocket of ERβ showing the H-bonds (---, point out with a red arrow) between the ligand and LBC. Regions of the protein are represented as red for negative, blue for positive, green for nonpolar, cyan for polar, grey for neutral. Amino acid residues interacting with the ligand via H-bonds are shown. All amino acids residues within 3 Å of the ligand are also shown to indicate the chemical nature of the binding pocket.

1.2.6 The distribution of ERs

Estrogens mediate the growth, bio-functions of many target tissues by activating an intranuclear binding protein (i.e., ERs).[52] The most common target tissues are in the male and female reproductive systems, such as the mammary gland, uterus, ovary, testis, and prostate (Fig. 1.9).[52] Estrogens are mainly produced in the ovaries and testes.[53] They diffuse in and out of all cells, but are retained with high affinity and specificity in target cells by ERs.[53] Once bound by estrogens, the ERs undergo a conformational change, allowing the receptor to bind with high affinity to chromatin and to modulate transcription of target genes.[14] In addition, estrogens also play an important role in bone maintenance and in the cardiovascular system, where estrogens have certain cardio protective effects.[12]

The biological significance of the existence of two ER isoforms is unclear. Perhaps the existence of two ER isoforms provides, at least in part, an explanation for the selective actions of estrogens in different target tissues which contains different expression levels of different ER isoforms (Fig. 1.9).[51] In fact, the high degree of interspecies conservation of the individual ER isoforms throughout vertebrate evolution could suggest that the basis for the selective effects of estrogens resides in the control of different subsets of estrogen-responsive promoters by the two ER isoforms.[51] This would indicate different expression of the ER isoforms in target tissues.[51]

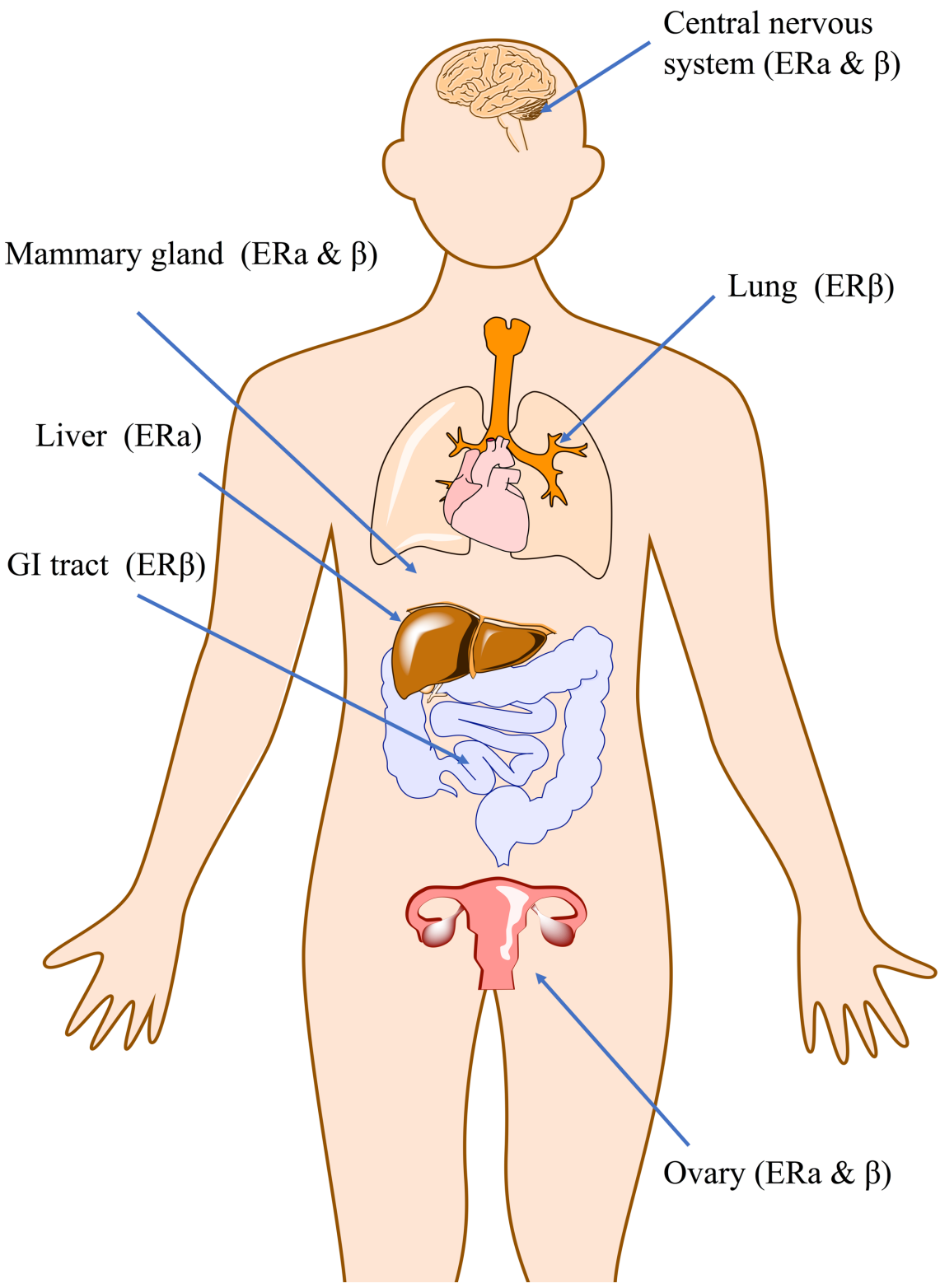


Figure 1.9: The distributions of ERs in the human body.

1.2.7 Evolution of ER: birth of the estrogen mimic

ER evolved in a pristine environment, in which it developed an intimate and highly specific relationship with estrogens, particularly E2.[54, 55] The specificity of this relationship was key to female sex hormone function.[54, 56] In time, the earth became polluted with chemicals, some of which have molecular analogues with E2.[54, 17] These estrogen analogues can occupy the previously estrogen-specific LBC and thus set the receptor off on its gene regulatory path.[54, 20] These estrogen analogues are termed estrogen mimics or xenoestrogens (from the Greek ξένος, meaning foreign) and are now thought to be responsible for human and ecosystem effects such as reduced human sperm count, precocious puberty,[54, 57] decreased alligator penis length, and imposex in dog whelks (*Nucella lapillus*)[58]— and all because of some man-made pollutants (e.g., the antifungal methylparaben used in some cosmetics; Fig. 1.10).[54,59] The first synthetic estrogen was produced in 1938, when Dodds's research group synthesised diethylstilbestrol (DES) (Fig. 1.10), a chemical compound with significant estrogenic properties.[54,60] In the study by McLachlan *et al*., DES was used to animal husbandry with effects such as increased weight gain in cattle, and caponisation of roosters.[61] Furthermore, DES has been used in medicine; for example, as a treatment for prostate cancer and for lactation suppression, and to reduce miscarriage rates.[62] In addition, some molecules derived from food plants (e.g., GEN from soybeans) are estrogen mimics;[54,17] because of this, soy

phytoestrogens have been applied in food supplements to alleviate menopause symptoms (e.g., hot flushes and loss of bone).[13]

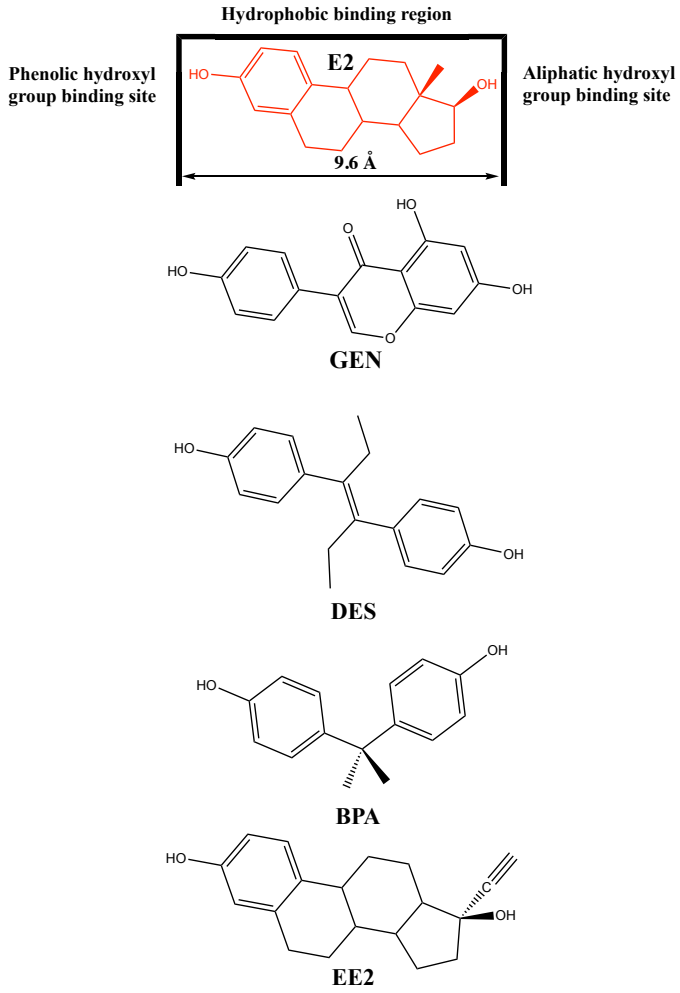


Figure 1.10: Schematic representation of ERα with E2 showing the ideal molecular requirements for binding. A selection of estrogen mimics is also shown, orientated to highlight their structural analogies to E2 and their potential interactions with the LBC.

1.3 Phytoestrogens and their implications for human health

1.3.1 Overview

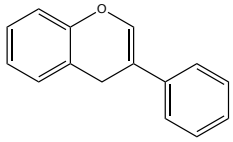
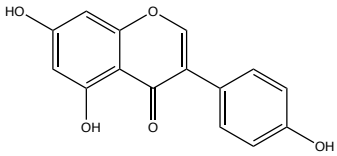
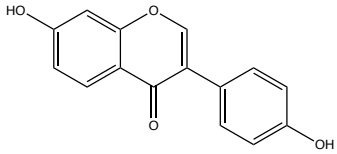
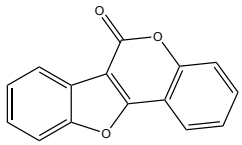
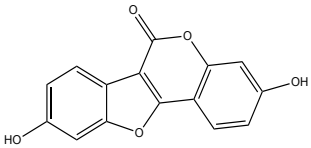
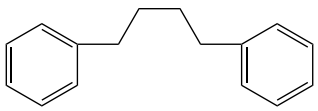
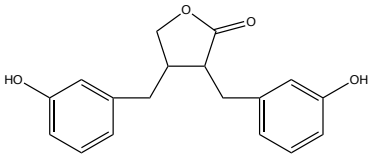
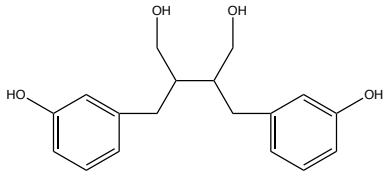
Xenoestrogens have been separated as phytoestrogens and synthesised xenoestrogens, depending on their sources.[63] Synthesised xenoestrogens come mainly from pharmacological estrogens (e.g., EE2, Fig. 1.10) and plastic containers (e.g., bisphenol A (BPA), Fig. 1.10).[64]

Phytoestrogens, however, are from plants, and have been found widely in dietary produce including fruit and vegetables.[65] Previous research shows that phytoestrogens possess many positive effects on human health; for example, they have anti-cancer and anti-oxidative properties in cell and animal model studies, and play an important role in the nervous system.[8, 66] However, some negative effects of phytoestrogens have been reported—chiefly but not limited to male feminization cryptorchidism, and precocious puberty in females.[67]

1.3.2 What are phytoestrogens?

The word "phytoestrogen" is derived from the Greek φυτόν meaning plant and estrogen, this means estrogen mimics from plant.[68] The major classes of phytoestrogens of current interest from a nutritional and health perspective are the flavonoids, coumestans, and lignans (Table 1.1), which are ubiquitous within the plant kingdom. They are biosynthesised in plants from phenylpropanoids and simple phenols. A conspicuous feature of the chemical structure of phytoestrogens is the presence of a phenol group, which is a prerequisite for binding to the ERs;[69, 70] The relative estrogenicity of these phytoestrogens (where E2 equals 1) is at least 10^4 times less than E2 (Table 1.1).[71, 72] Indeed, phytoestrogens can interact with ERs and trigger estrogen bio-actions; their actions at the cellular and molecular level are influenced by many factors, including but not limited to concentration dependency, receptor status, presence or absence of endogenous estrogens, and the type of target organ or cell.[73]

Table 1.1: Examples of phytoestrogens from three main classes and their estrogenic potencies,[72] where E2 equals 1.

Basic structure	Compounds	Relative estrogenicity to E2[71, 72]
Flavonoids 	GEN 	2.6×10^{-4}
	DAID 	1.1×10^{-4}
Coumestans 	Coumestrol 	3.0×10^{-4}
Lignans 	Enterolactone 	1.0×10^{-6}
	Enterodiol 	1.0×10^{-7}

1.3.3 Some examples of phytoestrogens

Coumestrol is a natural compound in the class of coumestans, and was first identified and reported as a phytoestrogen in *ladino clover* in 1957.[74] In addition, coumestrol has been detected in some foods, including soybeans, brussels sprouts, and spinach.[73] The highest concentrations of coumestrol were found in clover, kala chana (a type of chick pea), and alfalfa sprouts.[75] Coumestrol has been reported to increase the expression mRNA in the hypothalamus in brain which links the nervous system to the endocrine system *via* the pituitary gland; this indicates the potential role of coumestrol in modulating of endocrine.[76] Enterolactone and enterodiol are mammalian lignans formed by the action of intestinal bacteria from plant lignan precursors (e.g., matairesino and sesamin) present in the diet (e.g., whole grains and oil seeds).[77] Previous investigations indicated enterolactone and enterodiol may protect against breast cancer and prostate cancer;[95, 65, 78] besides the estrogenic-related health effect, the two phytoestrogens have been shown to be effective antioxidants against DNA damage and lipid peroxidation.[79] A good example of a flavonoid phytoestrogen is GEN from soy (Fig. 1.10).[80] GEN has a significant structural analogy with E2 (Fig. 1.10),[72] which allow this compound to bind with key amino acid residues in the LBC of ERs, *via* noncovalent interactions (e.g., hydrogen bonds (H-bonds), thus initiating the estrogenic response.[81] However, the phytoestrogens from different chemical classes show different estrogenicities because of their varying structures (Table 1.1).[72]

There are many health effects of phytoestrogens have been reported both in a positive and negative sense.[82] Positive effects include their use in post-menopausal women as a "partial hormone replacement" that could alleviates symptoms of the menopause when plasma E2 levels decline rapidly.[83] This rapid decline of E2 has been linked to a reduction in the incidence of post-menopausal disorders such as osteoporosis.[84] On the other hand, their negative health effects have also been reviewed extensively,[85-87] and are chiefly, but not limited to, male feminization (e.g., reduced sperm count,[88, 89] increased incidence of hypospadias[47] and cryptorchidism, precocious puberty in females,[90] as well as myriad effects in the environment, such as reduced penis length in alligators).[91]

1.3.4 The effects of phytoestrogens on health

1.3.4.1 Phytoestrogens and cancer

Traditional consumption of soy products has been linked to the lower incidence of breast and prostate cancers in Asian countries such as China and Japan compared with Western countries.[92] The potential association between dietary phytoestrogens and breast cancer has been studied frequently.[66, 93-95] At a cellular level, phytoestrogens including a total of 13 compounds from isoflavones, flavonoids, and lignans have been studied in previous research, and the results showed a biphasic effect of phytoestrogen on the DNA synthesis in human breast cancer cell (MCF-7 cells), which was inhibited at high concentration when the cells cultured with the selected phytoestrogens, but DNA synthesis in MCF-7 cells was induced exposure to low concentrations of the tested phytoestrogens.[96] Importantly, the induction effects of phytoestrogen on DNA synthesis is close to the probable level in humans.[97] Interestingly, in an *in vitro* study, the low concentration of GEN and DAID also showed antagonist inhibition of an anti-breast cancer medicine—tamoxifen (Fig. 1.11).[98] In addition, an *in vivo* experiment reports that rat were treated neonatally with GEN; and the results show that GEN treatment reduced the cell proliferation in the mammary gland.[98] In an epidemiological study of colorectal cancer (in 1997–2000), dietary phytoestrogens have been shown to be associated with colon cancer by reducing the risk of this cancer; this could be linked to the ERβ-mediated activity of such phytoestrogens in the colon which might influence the proliferation of colon cells.[66, 99 100]

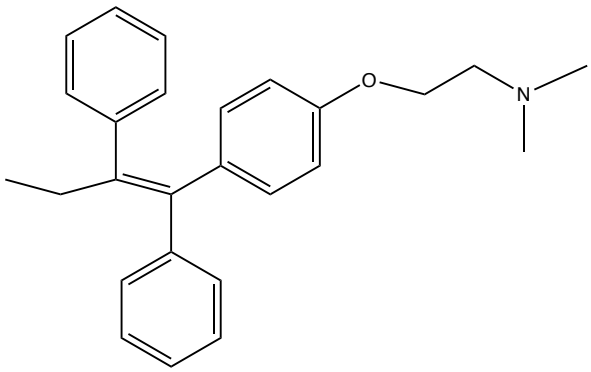


Figure 1.11: Molecular structure of tamoxifen.

ERs exist in the human GI tract (Fig. 1.9) and human colon cancer cell lines (e.g., Caco-2, T84, SW1116).[51] Some other epidemiological studies and trials consistently report that hormone replacement therapy (HRT) could cause a significant reduction in colorectal cancer risk among women.[101] A previous study suggested that estrogens could modify lipids and bile acid or decrease the estrogenic regulation, which might be associated with carcinogenesis; in turn, these estrogens may affect colorectal cancer risk.[51] Interestingly, a previous study indicated the estrogen inactivation in colonic cancer; the research indicated that E1 inhibited colonic cell proliferation, whereas E2 did not.[102] This means that the protective effect of HRT on colonic cancer might be due to estrone.[102] Several epidemiological studies indicated lowered colorectal cancer risk associated with the consumption of soy foods, although findings are varied by types of soy foods, colorectal cancer sub-site, and sex.[100] A case-control study evaluated the association between phytoestrogen (including lignan and isoflavone) intake and colorectal cancer risk.[99] The results showed that both dietary lignan and isoflavones intake was associated with a significant reduction in colorectal cancer risk.[99] In addition, the research also reported that encode enzymes (e.g., CYP450s, catechol O-methyl transferase) could be involved in the metabolism of phytoestrogens;[103] this suggests that dietary intake of phytoestrogens is potentially modifiable.[103]

1.3.4.2 Phytoestrogens and reproduction

Phytoestrogens can both promote and inhibit reproductive processes.[104] A previous research shows, phytoestrogens extracted from green tea, are able to inhibit cell proliferation, increase the rate of apoptosis, and influence the release of steroid hormones in porcine ovarian cells.[78] In addition, the development of precocious puberty and ovarian function might be altered by consumption of soybean-based foods; this is likely due to the high levels of phytoestrogen isoflavones in soy foods.[78] Another investigation in rats showed that, after 10 d of a soy-based diet in the postnatal period, offspring rats suffered from a high rate of demasculinization in adulthood.[105] Nevertheless, there are some studies showing adverse influences of xenoestrogens on sperm parameters; for example, previous research in the United States[60, 106] suggested that phytoestrogens could affect sperm quality.[60, 106] In addition, Shaw suggested that the food-derived xenoestrogen cocktail would have a greater effect on males because it adds a larger proportion to the total estrogenicity load.[107]

1.3.4.3 Phytoestrogens and cardiovascular disease

Coronary heart disease (CHD) is the leading cause of death in humans.[66] It has been suggested that a major factor for the increase of the incidence of CHD is the loss of E2 after menopause.[108] The abundance of certain phytoestrogens (e.g., soy isoflavones) in Asian diets and the lower rates of "Western diseases" such as CHD in such a population have suggested a protective role for these mostly soy-derived substances.[66] Phytoestrogens may also have effects on pathophysiologic vascular processes such as lipid profile (reduction of low-density lipoprotein, cholesterol, and these effects could delay the progression of atherosclerosis.[65] In addition, an animal model study reported that dietary soy isoflavones (i.e., GEN and DAID) improved coronary vascular function, by enhancing the dilator response to acetylcholine of atherosclerotic arteries in female monkeys, and the incidence of coronary artery disease has been reported to increase after menopause.[109, 110] This suggests the dietary supplement of food phytoestrogen may apply as a viable alternative to traditional HRT (e.g., E2).[109]

1.3.4.4 Phytoestrogens and the immune system

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB), is a transcription factor that can initiate inflammation and immune responses.[111] This intracellular signalling pathway can be suppressed by soy phytoestrogens (e.g., GEN), which suggested the soy phytoestrogen might affect the immune system.[112] Indeed, there are several *in vivo* and *in vitro* studies supported that phytoestrogens have the potential to affect the immune system.[113] For example, GEN inhibits antigen-specific immune response in an *in vivo* study in mice and suppresses lymphocyte proliferation *in vitro*, which is a fundamental characteristic of the response of lymphocytes to antigenic stimulation.[114, 115] Based on this, GEN has been used as a treatment for immune diseases in animal mode system studies, and exhibited inhibitory effect on allergic inflammatory responses.[113]

1.3.4.5 Phytoestrogens and the nervous system

In 1971, Gorsk suggested that the central nervous system (CNS) is a target for sex hormones.[116] Previous studies using light microscopic immunocytochemical and

hybridization observed the cells with ERα, ERβ, and guanine nucleotide-binding protein (G-protein) coupled with estrogen receptor 1(ER1), which is the gene coding ERα, throughout the brain, from the most rostral regions of the forebrain to the cerebellum.[117]

In the learning and memory region of the brain, including the prefrontal cortex, nucleus acumens, and dorsal striatum, membrane-associated ERs are observed, and this finding could underline a mechanism for the rapid effects of estrogens on these regions.[118] The effects of estrogens on dopamine-dependent cognition likely result from binding at both nuclear and membrane-associated ERs, so elucidating the localization of membrane-associated ERs helps provide a more complete understanding of the cognitive effects of these hormones.[117 119]

Since phytoestrogen can mimic E2 and activate ERs, emerging experimental and clinical evidence shows that phytoestrogens could influence memory mechanisms, cognition, postural stability, fine-motor skills, mood, and affectivity.[120] In addition, phytoestrogens may exert a protective action against neurodegeneration and brain injury.[120] Hajiranhimkhan *et al.* showed soy isoflavones may affect neurons *via* both steroid receptor and 5-hydroxytryptamine receptor (i.e., through both estrogenic and serotonergic activities).[95 ,121] In addition, phytoestrogen flavonoids have been reported to express various pharmacological potentials and mechanisms of action on the catecholamine (e.g., dopamine and epinephrine) system in adrenal medullary cells and sympathetic neurons; this suggests that these flavonoids might influence neuronal responses, by affecting catecholamine synthesis and uptake.[78, 122] Interestingly, GEN (but not DAID) can enhance noradrenaline uptake by noradrenergic neuroblastoma cells.[95, 120] In contrast, DAID was shown to inhibit catecholamine synthesis and secretion induced by a physiological secretagogue, namely, acetylcholine.[95, 120]

1.4 Phytoestrogens in food – risks versus benefits

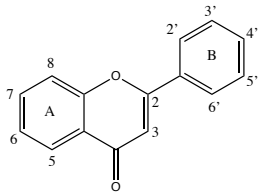
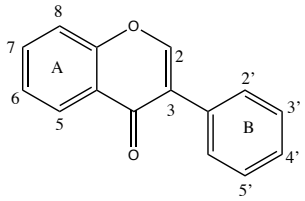
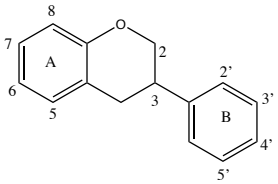
1.4.1 Overview

Phytoestrogens mainly come from flavonoids, which include a six main subclasses; flavone, isoflavone, isoflavane, flavanone, coumestan, dihydrochalcone. The dietary source of phytoestrogen flavonoids are fruits and vegetables, and soy isoflavones could constitute the majority, partly because of the huge consumption of soy and soy-based foods, and another reason is the relatively high concentration of isoflavones in soy (> 1%, dry weight) compared with other food sources of phytoestrogen flavonoids.[122] Soy-based foods are classified according to their food manufacturing process, namely, unfermented soy foods and fermented soy foods. The diverse food manufacturing processes; (soaking, grinding, filtration, and fermentation) could change the composition of isoflavones.[109]

1.4.2 Sources of phytoestrogens

As outline before, many phytoestrogens are flavonoids including six subclasses based on chemical structures (Table 1.2),[123] Isoflavones are the most common group of flavonoid phytoestrogens, and they are often found in legumes, such as soy beans.[123] There are two main compositions of isoflavones found in soybeans, daidzin and genistin, and their corresponding aglycones: DAID and GEN respectively (Table 1.3). They comprise more than 1% of the dry weight of soybeans, depending on the species;[124] for example, compared with American varieties of soybeans, Japanese varieties have greater amounts of isoflavones.[125]

Table 1.1: Examples of phytoestrogens from different subclasses of flavonoids, and their major food sources.

Subclass	Name	Substitution position	Food source
Flavone & Flavonol 	Apigenin	5,7,4′ (-OH)	Celery, parsley
	Acacetin	5,7, (-OH); 4′ (-OCH_3)	Black locust tree
	Kaempferol	5,2,7,4′ (-OH)	Grapes, onions
Isoflavone 	GEN	5,7,4′(-OH)	Soy-based foods (e.g., soy milk, tofu)
	Glycitein	7,4′(-OH); 6 (-OCH_3)	
	DAID	7,4′(-OH)	
Isoflavane 	*S*-equol	7,4′ (-OH)	Gut-mediated product of DAID

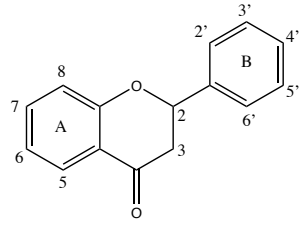
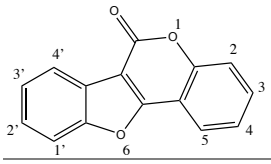
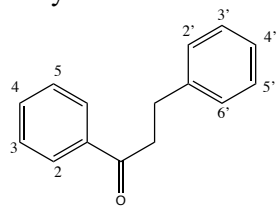
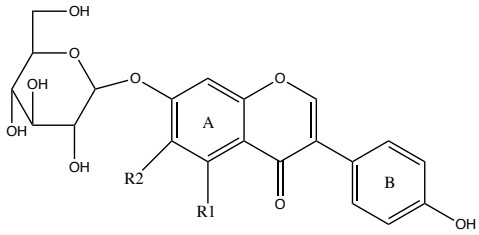
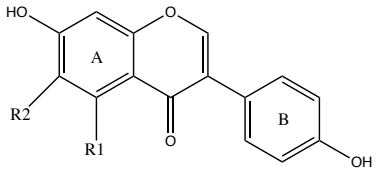
Flavanone 	Naringenin	5,7,4′(-OH)	Grapefruit, coconut
Coumestan 	Coumestrol	3,2′ (-OH)	Soy sprouts, lima beans
Dihydrochalcone 	Phloretin	2,4,6′,4′ (-OH)	Apple

Table 1.2: The main isoflavones in soy, and their structures.

Molecular forms	Substances	Substitutions	
Glucosides		R1	R2
	Daidzin	H	H
	Genistin	OH	H
	Glycitin	H	OCH_3
Aglycone	DAID	H	H
	GEN	OH	H
	Glycitein	H	OCH_3

1.4.3 Worldwide cultivation and consumption of soybean

Soybeans, indigenous to China, are one of the top five plant foods in China along with rice, millet, wheat, barley and have been cultivated for about 5000 years.[122, 126] Initially, soybeans were introduced to Southeast Asia, before being cultivated in Europe in the eighteenth century and America in the nineteenth century.[126] Since 1940, soybeans have been became one of the most crucial economic crops in the US, which is now the largest producer of

soybeans worldwide.[122] In addition, approximately 102.77 million hectares are used for soybean cultivation worldwide, with a total harvest of approximately 239.26 million tons in the 2011/2012 season.[122]

The nutritional value of soybeans has been documented widely.[127] Firstly, according to Zambiazi's research, soybeans contain significantly high levels of unsaturated fatty acids, particularly omega-3 fatty acids, which are well known to be an important member of the healthy fatty acids, and associated with cancer prevention, brain development and visual function.[128, 129] In addition, previous study concluded that soy-derived products are more beneficial than animal products, due to the lowering of serum triglycerides, low-density lipoprotein (LDL) and cholesterol of soy protein.[130] With a better understanding of soybean-related health benefits and consumer acceptance, soy products now account for a much larger share of the food market than ever before.[131] For instance, between 2000 and 2007, there were more than 2700 kinds of soy-based food product introduced into the US market.[131]

1.4.4 Unfermented soybean foods

Generally, soybeans foods are classified as one of two types, depending on the food manufacturing process: unfermented or fermented.[132] In Western countries, soybean foods, normally unfermented soybean foods, are an important component added in food manufacturing processes; for example, bread manufacturing.[132] There are various traditional soybean foods that have been widely accepted for a long time, for example, soybean flour and soy nuts.[132] Soybean flour is made from roasted soybeans that have been ground into fine flour; they are characterised by their fat content: either full fat or defatted.[122, 132] To increase the total protein percentage of foods, soybean flour is widely used as a basic ingredient in food production, especially in bakery goods and pasta; this is due to the significant amount of soy protein in soybean (soy protein constitutes about 50% of the dry weight of soybean).[133] Owing to major changes in food process technology, soy protein, either alone or in combination with some other vegetable proteins[122] is included in the production of various kinds of texturized meat substitute products, cheeses, and dairy substitute products.[133] These foods can enrich the diversity of vegetarian and medically necessary diets; for example, in the

management of hyperlipidaemia, which requires patient to decrease their intake of cholesterol.[133]

In the Eastern world, unfermented soybean foods have accounted for a large percentage of the diet since ancient times.[132] For example, soymilk is produced by soaking dried soybeans and grinding them in water, this food is in a written record in China from AD 82.[132] The aqueous extract of soy protein contains similar concentrations of protein and fat as unpasteurized cow's milk, but soymilk does not contain any lactose or cholesterol and is used as an alternative to dairy milk for lactose-intolerant people.[122, 134] Tofu is another popular unfermented soy food, also invented in China and introduced into Japan in the Tang Dynasty (around AD 618), and was introduced to Western countries only approximately 200 years ago.[135] Tofu is made by coagulating soymilk and then pressing the resulting curds into blocks.[24, 136] During the coagulating step, a coagulant (such as a calcium or magnesium salt or glucono delta-lactone) is used to precipitate soy protein from soymilk to form a jelly-like curd.[122, 137] Following the downstream processes, curds are converted into diversified intermediate products in different shapes.[137] Furthermore, to create diverse kinds of textures in terms of hardness, moisture, the jelly-like curds are further pressed and treated with food additives (e.g., soy sauce, chilli sauce).[138]

1.4.5 Fermented soybean foods

There are a variety of fermented soybean foods with a long production history and significant popularity in Eastern countries.[139] Miso is a very popular and widely used cooking condiment in Japan.[139] This soy-based food is prepared from soybeans, wheat, rice, and barley, alone or mixed with sea salt, and then fermented in a sealed container.[140] Generally, *Aspergillus oryzae*, yeast, and lactic acid-producing bacteria are required for the fermentation process.[140]

Soy sauce or Jiang You is one of the most commonly used seasonings in Eastern countries and has become popular in the Western world in recent year.[141] Soy sauce production has a similar ingredients and process to miso,[142] and soy sauce is made by mixing soybeans and roasted grain with mould cultures, such as *A. oryzae* and other microorganisms and yeasts.[143]

Soy sauces are classified according to their uses, such as laochou used for colouring, and shengchou used for seasoning with a shorter fermentation compared with laochou.[143]

Tempeh is a traditional Indonesian food produced by growing Rhizopus on peeled and partially cooked soybeans.[141] Typically, banana leaves are used as the fermenting cover. [141] Tempeh provides high levels of protein, and has been suggested as an important source of vitamin B13 and is used as a meat replacement in vegetarian diets in South Asia.[141]

Douchi (Natto in Japanese) is the first fermented soy food on record.[144] It originated in China (around 165 BC) and has been used as a food seasoning.[144] There are two types of douchi owing to the different strains of microorganism used in their fermentation, namely, *Mucor* sp., and *Aspergillus* sp., which is the most popular type of microorganism for soy fermentation.[144]

Sufu, also called Fu-ru or Dou-fu-ru, is a cheese-like soybean food with a soft texture and pronounced flavour.[145] Sufu is a side dish consumed normally with rice porridge in the south of China; in north China, people prefer to eat sufu with steamed buns.[146] Sufu is produced by fungal or bacterial fermentation of tofu.[147] The primary steps are the preparation of pehtze (tofu with culture mould), salting and ripening.[147] The various types of sufu are determined by their processing methods, colours and flavours.[146] For example, according to which fermentation method is used, there are naturally fermented sufu, bacterial fermented, and enzymatically ripened sufu[147] and the naturally fermented sufu is fermented slowly with naturally-occurring bacterial and fungal spores in the air.[146]

Doenjang is a popular fermented soybean paste from Korea, and can be used as a dipping agent for vegetables or barbecued meats.[148] This soybean paste is traditionally produced from meju, a fermented block made from crushed cooked soybeans.[149] The bacterium used in meju fermentation is *Bacillus subtilis*, and moulds such as *Mucor sp*, and *Aspergillus sp*. After the fermentation (1−3 months), depending on the block size, meju is set aside for further fermentation under brine in pottery jars.[149] The liquid and solid are separated after fermentation, with the solid residue being doenjiang.[150]

1.4.6 Effects of food manufacturing processes on the composition of isoflavones

Isoflavones exist as aglycones, including DAID and GEN, and as their corresponding glucoside conjugates in soybean and soybean-based foods (Table 1.3).[124] Many studied show that the compositions of isoflavones in soybean products would be changed during the manufacturing processes.[151] A good example is the production of tofu, the raw soybeans being soaked, ground with a certain proportion of water, then filtered, after which the supernatant was boiled and coagulated.[152] Previous research indicated that different ratios between water and soybeans during grinding varied the compositions of isoflavones.[152] Results showed the optimum water to bean ratio during grinding, in terms of estimating the loss of isoflavones, was 10:1.[152] It has been reported that heat treatment during the boiling could convert some malonyl isoflavones to their corresponding acetyl form, and a proportion of isoflavones were removed during defoaming in tofu production.[153] Another research studied the effects of coagulants on the isoflavone levels during tofu production. This study shows calcium sulphate is the best coagulant for tofu manufacture in terms of its high yield and preservation of isoflavones.[154]

Interestingly, fermentation has been reported to have a significant influence on compositions of isoflavones in soy foods.[147] The study of Yin *et al*. shows that most glucoside isoflavones are converted into their corresponding aglycone compounds during the fermentation process (Table 1.4).[147]

Table 1.3: Isoflavone compositions of sufu processing on its catalysis isoflavones content.[147]

Sample	Glucoside form (%, w/w)				Aglycone form (%, w/w)			
	Daidzin	Glycitin	Genistin	Total isoflavone	DAID	Glycitein	GEN	Total isoflavone
Raw soybeans	53.1	3.8	41.8	98.7	0.4	0.2	0.7	1.3
Tofu	20.1	2.7	59.2	82.0	13.6	3.6	0.8	18.0
Pehtze	2.5	1.2	15.5	19.2	28.7	5.6	46.5	80.8
Salted pehtze	2.5	0.8	12	15.3	30.8	6.1	47.9	84.7
Sufu	ND	0.3	NDa	0.3	33.5	7.8	58.4	99.7

ND, not detected

What is more, the activity of β-glucosidase, which catalyses the cleavage of the glucoside moiety to release the aglycone, was very low in tofu, but quickly increased during the fermentation; as a result, studies have shown highly significant increases in aglycones during fermentation.[11, 144, 147]

1.5 Metabolites of DAID and GEN during fermentation, and their biological activities

1.5.1 Overview

The fermentation of soy-based foods involves fungi, bacteria and yeast, which might cause biotransformation of isoflavones because of the enzymes released by these organisms, such as CYP450 and β-glucosidase.[155] A few hydroxylated metabolites have been found in fermented soy foods, and these compounds have been reported to have anti-cancer and higher anti-oxidative properties than their parent compounds.[156]

1.5.2 Hydroxylated compounds of GEN and DAID

An interesting point that should be appreciated from the research by Yin *et al*. is that the decrease of glucoside isoflavones (e.g., daidzin and genistin) was larger than the increase of their corresponding aglycones (e.g., DAID and GEN, respectively). [147] This suggests the aglycones might be further converted into other metabolites. Besides the conversion between glucoside isoflavones and aglycone isoflavones, two types of biotransformation products of DAID and GEN, namely ortho-hydroxydaidzein (OHD) and ortho-hydroxygenistein (OHG) have been isolated and identified from fermented soybean foods.[157] Only 3′-ortho-hydroxylated compounds are found naturally in plants as well as in fermented soy foods, such as *Orobus tuberosus*, *Machaerium villosum*.[157] These metabolites have the same structural skeleton but extra hydroxyl groups compared with their parent compounds.[157] The isoflavone metabolites are characterised by the position of the extra hydroxyl groups compared with their precursor compounds: OH-DAIDs and OH-GENs are named as 6-hydroxydaidzein (6-OH-DAID), 8-hydroxydaidzein (8-OH-DAID), 3′-OH-DAID (3′-hydroxydaidzein), 6-hydroxygenistein (6-OH-GEN), 8-OH-GEN (8-hydroxygenistein), and 3′-hydroxygenistein

(3′-OH-GEN) (Fig. 1.12). Meanwhile, their biological potencies have been determined by *in vivo* and *in vitro* experiments.[157]

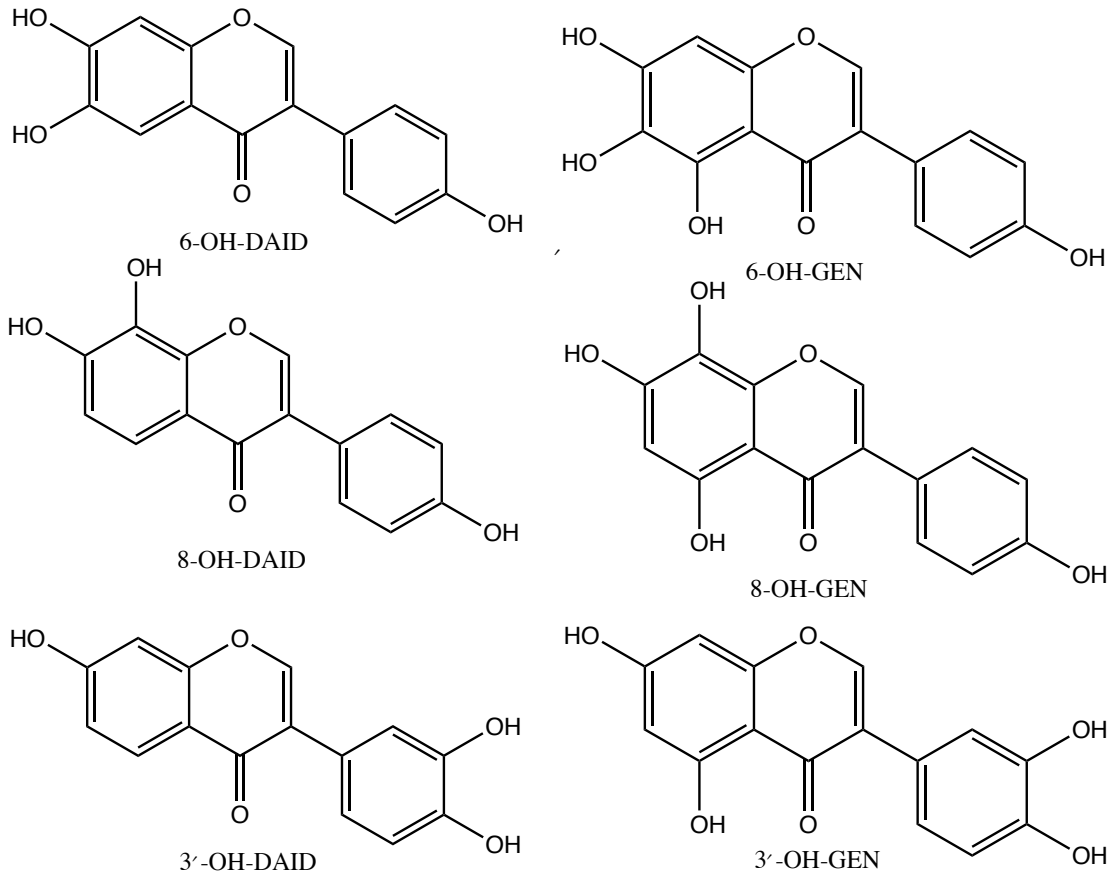


Figure 1.12: Structures of the hydroxylated derivatives of DAID and GEN produced during soy fermentation.

1.5.3 The sources of hydroxylated compounds of isoflavones and their bioactivity potency

6-OH-DAID was isolated from the Japanese soybean food koji, which is fermented with *A. oryzae*.[156] After soaking and steaming, soybeans were formed into cylindrical balls, and incubated at 30℃ for 4 d. In an *in vitro* experiment, 6-OH-DAID showed greater anti-oxidant functionality than DAID and GEN in a liposome test system.[156] In addition, 6-OH-DAID has

been reported to induce cell cycle arrest at the S and G2/M phases and suppress the proliferation of HCT-116 (a human colon cancer cell line). This DAID metabolite also exhibited a strong competitive activity in tyrosinase inhibition in both *in vitro* and *in vivo* experiments, this suggests the potential utilisation of 6-OH-DAID for skin disorder.[158, 159]

8-OH-DAID and 8-OH-GEN were isolated from miso fermented with *Aspergillus saitoi* and purified by silica gel column chromatography.[160] Previous study shows these two hydroxylated metabolites of isoflavones have significantly greater anti-oxidative activity in both oil-based and aqueous systems compared with GEN and DAID.[159] In addition, both of the metabolites showed tyrosine kinase inhibitory activity, and also exhibited anti-proliferative activity in cancer cell culture experiments: for example, HL-60 cell (human leukemic cell line) and HCT-116 cell (human colon cancer cell line).[139, 161, 162]

3′-OH-DAID was also isolated from miso and douchi, which are fermented with *Aspergillus oryzae* and *Salmonella. typhimurium* TA 98 at room temperature.[163] Both *in vitro* and *in vivo* experiments indicated that endothelial growth factor receptor-positive skin cancer cells can be suppressed effectively by 3-OH-DAID.[164, 165] In addition, the 3-OH-DAID showed relatively higher anti-oxidant potency by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay compared to its parent compound (i.e., DAID).

3′-OH-GEN was the first hydroxylated metabolite of isoflavones to be isolated from fermented soybean-based food (i.e., tempeh).[166] In bioactivity assays, 3′-OH-GEN induced G2-M cell cycle arrest and inhibited the proliferation of T47D breast cancer cells.[166] In addition, 3′-OH-GEN has been reported to against human immunodeficiency virus-1 (HIV-1) integrase and suggesting this compound may be used as a potential treatment for HIV1 infection (Acquired Immune Deficiency Syndrome (AIDS).[149] Moreover, 3′-OH-GEN shows extremely potent of hepatoprotective activity on D-galactosamine-induced cytotoxicity in primary cultured mouse hepatocytes.[167-169]

1.6 Aim and objectives of this thesis

The overall aim of this thesis is study the complex interrelationships between ERs, their ligands, and bioactivities, focusing on isoflavones in a functional food setting. This will be achieved by the following research objectives:

- Use a computational bio-molecule platform (Schrödinger) to investigate the communication between the LBC and AF-2 of ERα *via* the conformation changes caused by docked ligands.

- Use Schrödinger to study the effects of structural properties of ligands (i.e., in this thesis flavonoids) on their theoretical ERα binding energy, and potential implication for estrogenicity.

- Use Schrödinger to investigate the theoretical binding energy/affinity of isoflavones with ERα.

- Use the MELN assay to study the structure ERα-driven bioactivity relationship of isoflavones.

- Use Schrödinger to study the theoretical binding energy and binding affinity of isoflavones with ERβ.

- Study the effects of isoflavones on the proliferation of Caco-2 cells, and investigate the potential structure-activity relationships of isoflavones.

- Investigate the potential influence of the intestinal phase II metabolism on the ERβ-driven bioactivity of isoflavones.

- Use an *in vitro* gut fermentation model to investigate the effects of isoflavones on human gut bacterial populations.

- Use a Caco-2 monolayer transportation system to study the absorption and metabolism of isoflavones.

1.7 Thesis map

The interrelationships between the chapters in this thesis are shown in Figure 1.13.

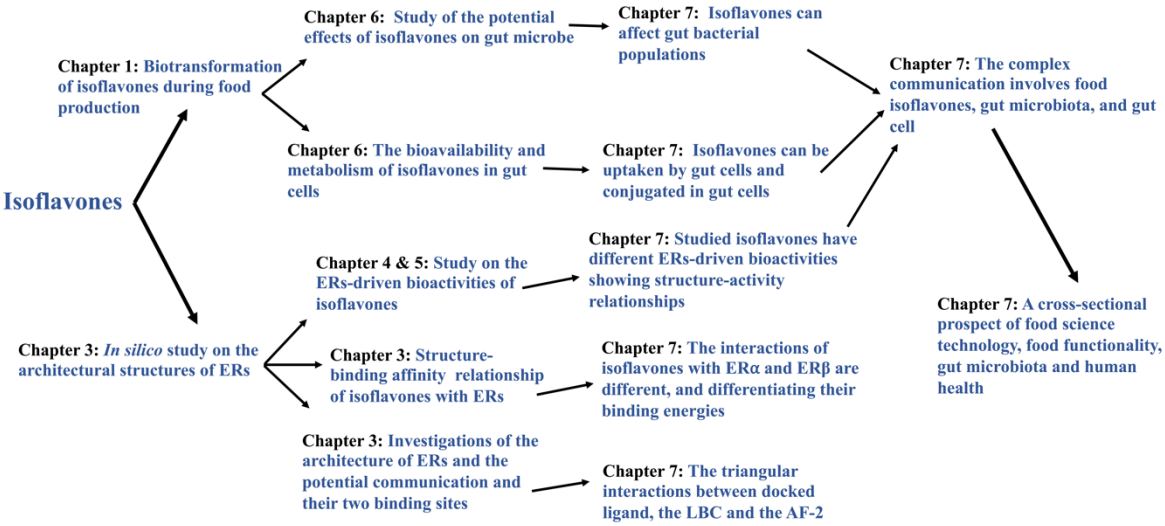


Figure 1.13: Schematic to show the interrelationships between the chapters in this thesis.

Chapter 2: Materials and Methods

2 Chapter 2 –Materials and Methods

2.1 Materials

2.1.1 Chemicals

2.1.1.1 General Chemicals and Solvents

All general chemicals were purchased from ECP Ltd, New Zealand, unless listed below.

Dimethyl sulfoxide (DMSO; Scharlau Chemie, SA, Spain).

Gallic acid (GA) (Sigma-Aldrich, New Zealand Ltd.).

High Performance Liquid Chromatography (HPLC) grade methanol, acetonitrile (Sigma-Aldrich, New Zealand Ltd.).

Milli-Q water (purified using the Milli-Q system, 18.2 MΩ/cm, Merck Millipore Merck Ltd., Auckland, New Zealand).

2.1.1.2 Authentic standards

6-hydroxydaidzein (6-OH-DAID), 8-hydroxydaidzein (8-OH-DAID), 3´-OH-DAID (3′-hydroxydaidzein), 8-OH-GEN (8-hydroxygenistein), and 3′-hydroxygenistein (3´-OH-GEN) (Indofine Chemical Company, Hillsborough, NJ, US).

17β-Estradiol (E2), genistein (GEN), daidzein (DAID), formononetin (FOR) (Sigma-Aldrich, St. Louis, US).

Fulvestrant (ICI 182,780, Sigma-Aldrich, St. Louis, US).

All the authentic standards were dissolved in DMSO to make 1 mM stock solutions.

2.1.1.3 Lab wares

Centrifuge tubes (15 mL, 50 mL; LabServ®, Thermo Fisher Scientific, New Zealand Ltd., Auckland, New Zealand).

Eppendorf tubes (100 µL, 600 µL, 1.6 mL; Thermo Fisher Scientific, Melbourne, Australia).

Glass HPLC vial (2 mL, SUN SRi, Thermo Fisher Scientific, Auckland, New Zealand).

Hamilton Syringe (10 µL, Sigma-Aldrich, New Zealand Ltd.).

Non sterile syringe filters (7.5mm non-sterile PTFE hydrophobic filter, Thermo Fisher Scientific, Melbourne, Australia).

Micro well white tissue culture plates, sterile (Nunc™ F96 MicroWell™ White Polystyrene Plate 24, 96 wells, Thermo Fisher Scientific, New Zealand Ltd., Auckland, New Zealand).

Periplast plastic counting chamber (Thermo Fisher Scientific, Melbourne, Australia).

T-75 sterile culture flask (Sigma-Aldrich, New Zealand Ltd.).

Sterile culture flasks (Nunc™ EasYFlask™ Cell Culture Flasks, 75 cm^2, filter, Thermo Fisher Scientific, New Zealand Ltd., Auckland, New Zealand).

Sterile filters (Steritop-GP, 0.22 µm, polyethersulfone, 500 mL 45 mm, Merck Millipore).

Varioskan Flash (Thermo Fisher Scientific, New Zealand Ltd., Auckland, New Zealand).

2.1.1.4 Biologicals

Benzyl penicillin (Sigma-Aldrich, New Zealand Ltd).

Bovine Serum Albumin (BSA; Sigma-Aldrich Ltd., New Zealand).

D-Luciferin (Sigma-Aldrich Ltd., New Zealand)

Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich Ltd., New Zealand).

Fetal bovine serum (FBS; Life Technologies, Auckland, New Zealand).

Inulin power (Sigma-Aldrich Ltd., New Zealand).

L-Cysteine HCl (Sigma-Aldrich Ltd., New Zealand).

LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics, Germany).

Minimal Essential Medium (MEM) with Earle's Salts and 20 mM L-glutamine (Sigma-Aldrich New Zealand Ltd).

Phenol red-free MEM powder (Sigma-Aldrich, New Zealand Ltd).

Radioimmunoprecipition (RIPA) buffer (Sigma-Aldrich Ltd., New Zealand).

Resazurin sodium salt (Sigma-Aldrich Ltd., New Zealand).

Streptomycin sulfate (Sigma-Aldrich, New Zealand Ltd).

Sulfatase/β-glucuronidase from Helix pomatia (≥10,000 units/g solid) (Sigma-Aldrich, St. Louis, US).

Trypan blue (Sigma-Aldrich, New Zealand Ltd).

Type-I collagen (Sigma-Aldrich, New Zealand Ltd).

TrpLE® express (Thermo Fisher Scientific, Auckland, New Zealand).

Trypsin powder (Becton Dickinson, Auckland, New Zealand).

Wilkins Chalgren anaerobic (WCA) broth (Thermo Fisher Scientific, Melbourne, Australia).

ZR Faecal DNA kit (Zymo Research Corporation, Irvine, CA, US).

2.2 Equipment

Anaerobic chamber (Coy Laboratory Products Inc., Michigan US).

Octadecylsilane (ODS) SPE bulk sorbent (Agilent, US).

C_{18} SecurityGuad column (Phenomex, North Shore City, New Zealand).

Centrifuge (multifuge 1 S-R, Heraeus, Hanau, Germany).

HPLC System (Dionex, US).

Incubator shaker (Thermo Fisher Scientific, Melbourne, Australia).

Inverted microscope (CKX41, Olympus, Melbourne, Australia).

Laminar flow cabinet (Cytoguard CG2000 series, model CGA-180, Clyde Apac, Sydney, Australia).

Microscope camera (Toup Ltd.).

Millipore sterile filter (0.45 µM, Merk Millipore, US).

Millicell voltohmmeters (Merck, Germany).

Phenomenex C_{18} Prodigy 5µm ODS3 100 A 250 × 4.60 mm column (Phenomenex, North Shore City, New Zealand (S/No 584453-48).

Rotor-Gene 6000 machine (Bio-strategy, New Zealand).

Transwell Insert (Life Sciences, MA, US).

Techne Sample Concentrator (Total Lab System Ltd, Auckland, New Zealand). Preparation of cell culture media, bacteria culture media and related reagents

Vortex mixer (DLAB, China).

2.2.1.1 Antibiotics

Benzyl penicillin (3.0 g) and streptomycin sulfate (2.8 g) were added to 100 mL sterile Milli-Q water; this mixture was stirred using a magnetic stirrer for 24 h. The final solution was stored at 4°C for up to six months.

2.2.1.2 PBS buffer

A packet of PBS powder was added to a 1 L Schott bottle. Milli-Q water (900 mL) was added and the PBS dissolved *via* vigorous shaking. The pH was adjusted to 7.4 using 1M or 0.1M HCL or 1M or 0.1M NaOH as appropriate. The pH adjusted solution was topped up to 1 L with Milli-Q water. The solution was autoclaved (120°C, 15 psi for 80 min) and then stored at 4°C.

2.2.1.3 Trypsin proteinase

NaCl (8.5 g) was dissolved in Milli-Q water (1 L). Trypsin powder (25 g) was added to the 0.85% w/v (aq) NaCl (1 L) and stirred (using a magnetic stirrer) at room temperature for 1 h. The trypsin solution was sterilised by filtration and dispensed into 10-20 mL aliquots and stored at -20°C. EDTA (3.72 g) was dissolved in PBS (1 L) and sterilised by autoclaving. This PBS/EDTA (PE) solution was diluted 10-fold with PBS and 90 mL of the diluted PE was mixed trypsin solution (10 mL) to produce the final trypsin solution (2.5% w/v, (aq). This solution was stored at 4°C for up to 3 weeks.

2.2.1.4 Heat inactivation of fetal bovine serum

Fetal bovine serum (FBS, 500 mL) was thawed at 4°C overnight. The thawed serum was gently warmed in a 37°C incubator for 30 min with gentle inversion every 10 min to ensure even temperature distribution. After the serum reached 37°C it was placed in a 56°C water

bath for 60 min with gentle inversion every 10 min. The serum was left to rest at room temperature for 30 min. Aliquots (100 mL) were transferred to Schott bottles and stored at −20°C.

2.2.1.5 Charcoal-dextran Stripped FBS

$MgCl_2$ $(H_2O)_6$, sucrose (85.6 g), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 2.4 g) was added to a 1 L Schott bottle. Milli-Q water (1 L) was added to the bottle and shaken vigorously. The solution was divided into 50 mL aliquots. Two aliquots were added to two 50 mL plastic centrifuge tubes each containing dextran coated charcoal (0.137 g). The centrifuge tubes were inverted 10 times to ensure even distribution of charcoal-dextran. The tubes were incubated for 24 h at 4°C.

Following incubation, the charcoal-dextran solution was centrifuged at 500 × g for 10 min and the supernatant was discarded. Inactivated FBS (50 mL) was added to each pellet. The tubes were inverted 10 times and incubated for a further 24 h. The FBS-charcoal mixture was then centrifuged at 1700 × g for 10 min and the stripped FBS decanted into a 100 mL Schott bottle and stored at −20°C.

2.2.1.6 Phenol Red RPMI-1640 Medium Containing 10% v/v Inactivated FBS

Heat inactivated FBS (100 mL) was thawed at 4°C overnight. Phenol red RPMI-1640 powder (9.6 g) and $NaHCO_3$ (2.0 g) were added to a 1 L Schott bottle. Milli-Q water water (900 mL) was added and the powder dissolved by vigorous shaking. The pH was adjusted to 7.4 (see ***Section 2.1.3.2***) and Milli-Q water (100 mL) was added. Sodium pyruvate solution (1 mL) was added to the RPMI-1640 solution and 900 mL was sterilised *via* ultra-filtration through a 0.22 µM filter. The antibiotics (5 mL) and FBS (100 mL) were sterilised *via* ultra-filtration through the same 0.22 µM filter, respectively. The complete RPMI-1640 culture medium solution (1 L) was stored at 4°C.

2.2.1.7 Phenol Red Free RPMI-1640 Medium Containing 10% v/v Stripped FBS

Charcoal-dextran-stripped FBS (100 mL) was thawed at 4°C overnight. Pre-bought phenol red free RPMI-1640 culture medium solution (900 mL) was sterilized *via* ultra-filtration using a 0.22 µM filter, followed by adding antibiotics (5 mL) and FBS (100 mL). The complete phenol red free RPMI-1640 culture medium solutions (1 L) was stored at 4°C.

2.2.1.8 Minimal Essential Medium (MEM) containing 10% v/v heat-inactivated FBS

Heat inactivated FBS (100 mL) was thawed at 4°C overnight. MEM (900 mL) was sterilized *via* ultra-filtration using a 0.22 µM filter, followed by antibiotics (5 mL) and FBS (100 mL), respectively. The complete phenol red free MEM culture medium solution (1 L) was stored at 4°C.

2.2.1.9 Trypan blue solution (0.4% w/v in PBS)

Trypan blue power (0.4 g) was dissolved in 100 mL PBS.

2.2.1.10 Luciferin stock solution

A stock solution of luciferin was prepared in DMSO (25 mg/mL). Aliquots (200 µL) were stored in amber vials at −80°C.

2.2.1.11 Inulin solution

Inulin powder (500 mg) was added to a 1 L Schott bottle. Milli-Q water (500 mL) was added and the powder dissolved by vigorous shaking. Then, the inulin solution was stored at 4°C.

2.2.1.12 Wilkins Chalgren anaerobic (WCA) broth

Wilkins Chalgren anaerobic (WCA) broth powder (33 g), L-cysteine HCl (0.5 g), resazurin sodium salt (0.5 mg) were added to a 1 L Schott bottle,[170] Milli-Q water (900 mL) was added. The pH was adjusted to 6.8 using 1 M and 0.1 M HCL or NaOH as appropriate. The pH adjusted solution was made up to 1 L with Milli-Q water. The solution was autoclaved (120°C, 15 psi for 80 min) and then stored at 4°C.

2.3 Autoclaving

All glassware and consumables including Schott bottles, glass pipettes, sample vials, micropipette tips and Eppendorf tubes were autoclaved at 120°C, 15 psi for 80 min. All autoclaved equipment was then dried at 75°C for 1 h prior to use.

2.4 Caco-2 cell maintenance and passage

Caco-2 cells were routinely passaged when the cultures has reached confluence (approx. 10^7 cells). Used MEM was vacuum aspirated using a flame sterilised Pasteur pipette. PBS (4 mL) was added to inactivate the any residual MEM. TrpLE® express (3 mL) was added to detach the cellular monolayer. The culture flasks were incubated at 37°C with 5% v/v CO_2 in air until cells were seen to be fully detached under an inverted microscope (approx. 10 min) MEM (10 mL) was added to inactivate the TrpLE® express and the cell suspension was transferred to a 50 mL centrifuge tube. The suspension was centrifuged at 4000 × g for 5 min and the supernatant was vacuum aspirated. The pellet was suspended in fresh MEM (10 mL for each new culture) and vortex mixed for 10 s to produce a homogenous suspension. Cell suspension (10 mL) was added to a 75 cm^2 culture flask containing 10 mL fresh MEM to give a total volume of 20 mL. The cells cultures were incubated at 37°C in air.

2.5 Cell counting

Vertiplast Plastic Counting Chambers were used to count large volumes of cells samples at any given time. An aliquot of cell sample suspension was collected at known volume (20 µL) and mixed in 1:1 ratio with trypan blue. A 9 µL aliquot of each sample was added to 9 × 9 grids (triplicate). The same 5 squares were counted for each sample under an inverted microscope at 100 × magnification.

The total number of cells per mL was calculated using the formulae:

$$C_{mL} = \frac{T \times 10^3}{k \times N} \qquad C_{total} = C_{mL} \times V\ mL$$

- $C_{mL} = Cells\ per\ mL$
- $C_{total} = Total\ number\ of\ cells\ in\ the\ cell\ suspension$
- $T = Total\ number\ of\ cells\ counted$
- $k = 0.01111$
- $N = 5$
- $V = Total\ volume\ of\ cell\ suspension$

2.6 Experimental Procedures

Specific details of the experimental procedures are described under “Experiments” in each chapter.

Chapter 3: *In silico* studies of ERα and its intimate interactions with ligands

3 Chapter 3 – *In silico* studies on ERα and its intimate interactions with ligands

The content of this chapter is the subject of the following publication: Ye H, Dudley SZ & Shaw IC (2018) Intimate estrogen receptor-α/ligand relationships signal biological activity. *Toxicology*. **408**, 80-87

3.1 Introduction

3.1.1 Overview

ERs, including ERα and ERβ, are nuclear receptors (NRs) which control the development, homeostasis and metabolism of the organism.[14] The LBD, which is one of the six functional domains on ERs; has two interacting binding sites LBC and AF-2. The LBC directly interacts with ligands can cause knock−on effects to facilitate the AF-2 to accommodate regulatory proteins.

3.1.2 Estrogen receptors and their mechanism of estrogenic activity.

NRs are transcription factors that control essential developmental and physiological activities.[171] Many NRs, such as ERα and ERβ respond to a hormonal and metabolic milieu that includes high affinity natural ligands such as E2 and an ensemble of lower affinity ligands such as environmental xenoestrogens (e.g., BPA).[172, 173] This can lead to diverse endocrine−disrupting health effects, including cancer, infertility, and development mental abnormalities.[174, 175] Both of ERα and ERβ are E2−activated nuclear receptors (NR3A1–nuclear receptor subfamily 3, group A, member 1 & 2).[54] As outlined above, the LBD is one of the functional domain of ERs, it comprises two separates, but connecting binding clefts: LBC and AF-2.[20] Under normal circumstances, E2 binds to the LBC and initiates a protein

conformational changes, exposing AF-2 to interaction with regulatory proteins.[54, 3] Phosphorylation of serine then occurs which aids the targeted receptor (e.g., ERα) dimerization.[21, 176] The dimer then moves into the nucleus and binds to DNA *via* a DNA binding domain (ERE) or *via* a protein DNA binding intermediate.[54, 177] Recruitment of regulatory protein then occur.[54, 178] Furthermore, regulatory protein recruitment controls the degradation of ERs *via* tyrosine phosphorylation.[54, 179] This phosphorylation signals initiation of the proteasome ubiquitination pathway, leading to polyubiquitinated ERs which are transported to the proteasome for degradation.[54, 180] The regulatory protein recruitment influences the ubiquitination process, leading to differential downstream bioactivity effects; for example, cell proliferation and cell migration.[54, 179] An amino acid core region in the ERs binding domains contain two zinc finger motifs that cooperate to stabilize a rigid DNA−binding recognition helix and a flexible helix supported dimerization loop which docks onto DNA's ERE.[19] This results in gene expression modulation[181] and ultimately cellular feminization (Fig. 3.1). In this chapter, the intimate "triangular relationship" between docked ligands, the LBC and the AF-2 will be explored and studied.

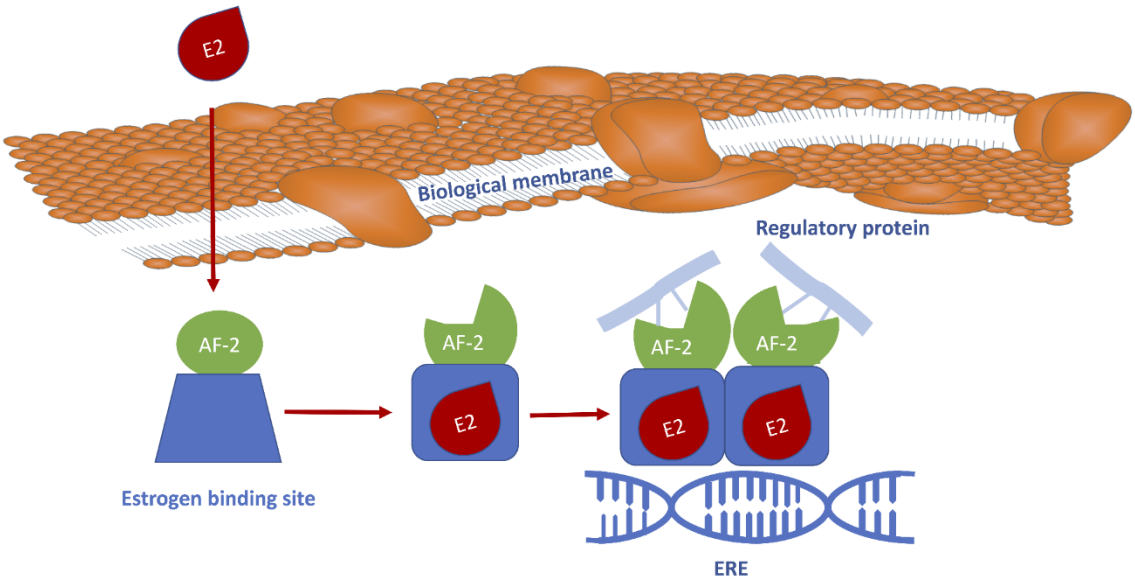


Figure 3.1: The process of E2 docking at the LBC of an ER resulting in a knock-on effect on AF-2 to accommodate regulatory proteins. E2 passes through the biological membrane then is docked in the LBC of the ER resulting in a conformational change; this interaction between the docked ligand and LBC trigger the knock-on effect on AF-2, and generates the binding interface for regulatory proteins. After the dimerization of ERα, the dimer bonds to ERE (Diagram by author).

3.1.3 The requirements for binding at the LBC

Highly efficient and specific binding of ligands to the LBC are prerequisites for the biological activity of estrogen action.[54] A decade ago, Müller *et al.,* illustrated the structural homology between different ligands bonded with the LBC, and suggesting the potential importance of hydroxyls of ligands in the their interactions with LBC (Fig. 3.2).[54, 182] In addition, the X-ray crystal structures of complexes of ERs with different ligands (e.g., E2 (PDB entry code: 1ERE), and GEN (PDB entry code: 1X7R) indicate the specificity of the interactions could be mediated by hydrogen bonds (H-bonds) between key functional groups on the ligands (e.g., E2's 17β-hydroxyl; Fig. 1.7) and specific LBC amino acid residues (e.g., His 524).[54, 20] This 3-dimensional lock and key approach exploits the unique spatial

arrangement of the ligand's functional groups and relevant amino acid residues in the LBC.[54, 20] Once a ligand is docked, it appears that hydrophobic interactions are created between the ligand and the LBC.[54, 20] This pulls ER's helices towards the ligand, initiating the all−important conformational change which facilitates receptor dimerization and consequent bioactivity.[54, 20, 183]

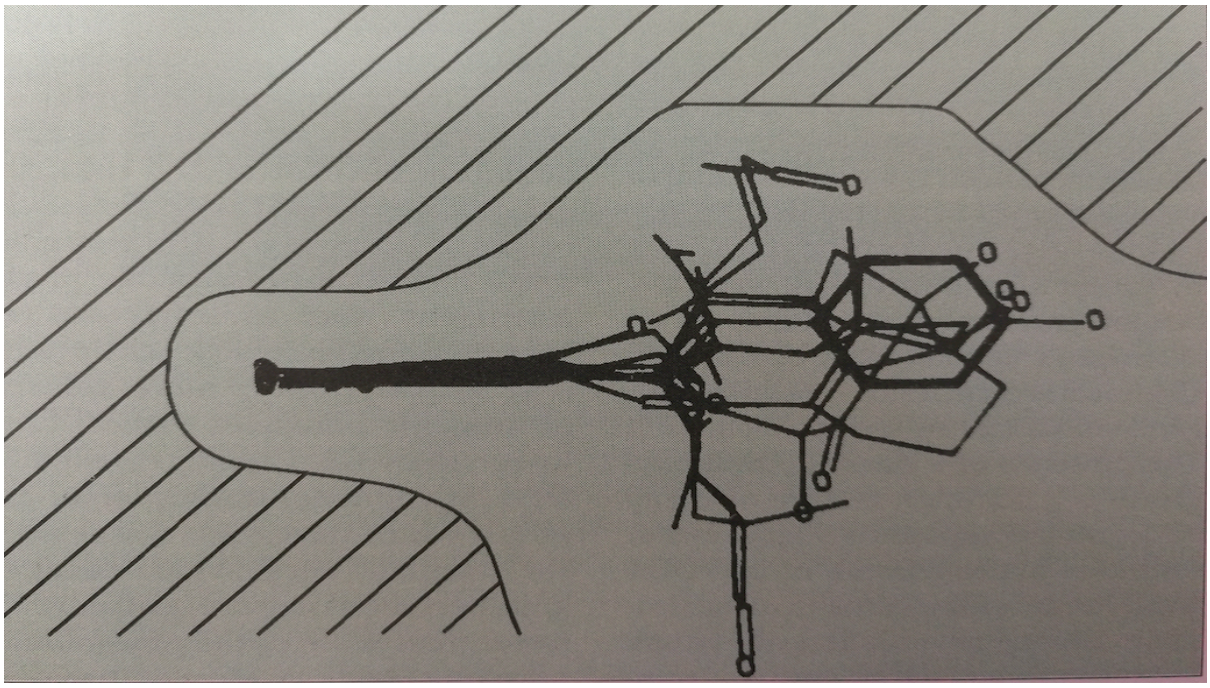


Figure 3.2: Superposed xenoestrogens and E2 in the LBC showing the structural similarity between xenoestrogens and E2. Figure from an early paper describing estrogen mimic interactions with ERs—a concept was born. Figure adapted from Müller *et al*., (1995) Toxicological aspects of oestrogen-mimetic xenobiotics present in the environment, Toxicol. Ecotoxicol. News, p 69 Fig. 1. Reproduced with permission of the publisher: Taylor & Francis.

The binding kinetics and Gibbs free energy of the noncovalent interactions at the interface between a ligand or a regulatory protein and its targeted binding site (i.e., LBC or AF-2), balanced by dissociation /ligand kinetics determine the lifetime of the binary complex and thus overall biological activity.[54, 184] The presence and strength of noncovalent interactions rely on the relative locations and spatial arrangements of specific amino acid residues which

facilitate the interactions with a ligand or a regulatory protein.[54, 64] Thus, the different ligands could have different binding energies and binding affinities with ERs,[54, 184] which differential their estrogenicities; and in turn, this might affect food functionality of phytoestrogen–containing foods (e.g., isoflavones in soy)—this is a focus of the research presented in this thesis.

3.1.4 Food phytoestrogens and their gut-mediated metabolites

Food functionality is gaining significant importance in a marketing context and is being advocated for the management and prevention of some disorders and diseases.[185] For example, isothiocyanate–rich foods (e.g., broccoli) have been promoted for the prevention of colorectal cancer, and resveratrol in red wine has been associated with life-prolonging, anti-cancer and cardio-protective properties.[186] Flavonoids are important food components, theoretically, they fit the binding characteristics of ERs (i.e. two aromatic hydroxyls separated by approximately the right length of hydrophobicity) (Table 1.1 & 1.2).[187] In addition, many flavonoids have been proved to be estrogen mimics and utilised in a functional food context; for example, isoflavones−rich breads are used as partial 'hormone' replacements for postmenopausal woman.[188] Chemically, flavonoids have the general structure of a 15–carbon skeleton, which consists of two phenyl rings (A and B) and heterocyclic ring (C) with various substitutes patterns in the different subclasses including isoflavone, isoflavane and flavone.[187] For example, isoflavone is ketone containing compounds, but isoflavane is not (Table 1.2). In addition, within the same subclass, the individual compounds have different hydroxyls arrangements (Table 1.2).[187] Both of these structural characteristics might affect bioactivity of flavonoids; for example, estrogenicity.

In addition, gut microbiome comprise tens of trillions of bacterial cells.[189] A major function of the bacteria is to process the metabolism of food functional compounds (e.g., flavonoids) and producing metabolites which can be utilised in beneficial ways to support host biology.[170] Two examples of the metabolites of food phytoestrogens are: DAID is

metabolised to the more estrogenic *S*-equol (Table 3.3, Fig. 3.3),[190] and GEN is converted to less estrogenic dihydrogenistein (Fig. 3.3).[191] However, the molecular basis of the different estrogenicities of metabolites compared to their parent phytoestrogens is still unknow.

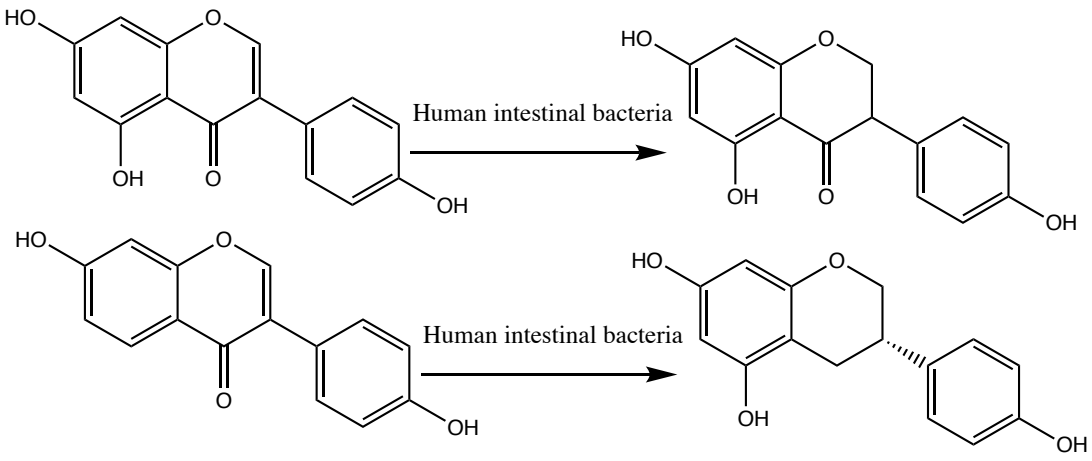


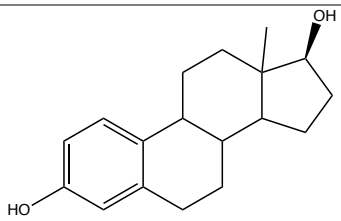
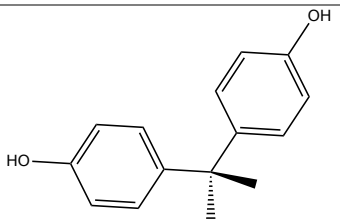
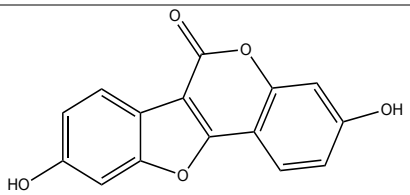
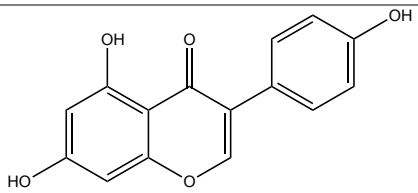
Figure 3.3: Top: gut microbiome−mediated biotransformation of GEN (left) to dihydrogenistein (right). Bottom: gut microbiome−mediated biotransformation of DAID (left) to *S*-equol (right).

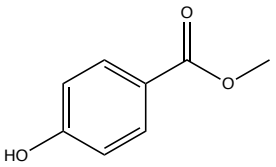
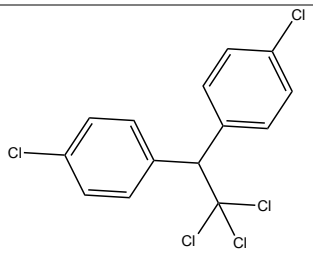
A better understanding of the mechanisms underlying the bioactivity of chemicals is crucial for any health claims to be made. However, such studies are time consuming and expensive. Therefore, the application of *in silico* modelling studies to investigate possible receptor−mediated mechanisms might be useful in exploring food component functionality. The incorporation of enhanced levels and extracts of active principles from functional foods in food supplements further illustrates the economic importance of food functionality in a health setting.[192] For example, resveratrol dietary supplements are marketed as preventing heart disease and cancer despite their being no clinical trials to support their efficacy.[192] An understanding of the possible mechanisms of biological activity of functional food components is important if they are to be developed in a health promotion setting. Some bioactive food components have been studied by the pharmaceuticals industry as potential drugs;[193] for example, resveratrol underwent clinical trials in 2010, but was withdrawn because of concerns about its side effects.[186] Others have never been extensively studied.

3.1.5 The advantages of *in silico* study

In the context of receptor—mediated biological activity, structure affinity relationship is the interdependency between compound structures and their binding affinity.[173] This is a good predictor of biological activity and toxicity, and is useful as a preliminary to deciding (in a drug development context) whether a new compound has pharmaceutical potential.[194] For example, results of a molecular modelling studies show gossypol (a natural polyphenol compound extracted from cotton seeds) can bind B-cell lymphocyte/leukemia-2 (Bcl-2) family proteins, such as B-cell lymphomaextra-large (Bcl-x_L), which is critical component of the intrinsic apoptotic pathway.[195] This suggested that gossypol is a potent inhibitor of Bcl-2 proteins, and thus gossypol was regarded as a potential chemotherapeutic agent.[196] Indeed, gossypol has been reported to target Bcl-2 family protein and inhibit a wide range of human carcinoma cell lines derived from breast (T47D), prostate (Du-145), cervix (HeLa).[196] This clearly showed the predictive power of molecular modelling in the context of receptor/ligand binding. In addition, E2 and its interaction with ERs have been studied *in silico* which gives a clear understanding of the molecular attributes necessary for estrogenic activity.[20, 197, 198] For example, comparing the structures of E2, dichlorodiphenyltrichloroethane (DDT) and BPA and their estrogenicities, which were determined by MCF-7 cell proliferation experiment, demonstrate this well (Table 3.1).[190, 202] The requirements for occupancy of ERs are an aliphatic hydroxyl group and an aromatic hydroxyl group (i.e., the potential form H-bonds) separated by 9.6 Å of hydrophobicity; E2 fulfils these requirements exactly because it is ER's natural ligand.[20] BPA partially fulfils the ideal binding requirements—it has two aromatic hydroxyl groups separated by 9.3 Å by a region of hydrophobicity;[199] however, DDT has chlorine atoms which do not form H-bonds but form "halogen bonds" due to their electronegativity which interact electrostatically with key amino acid residues in the LBC of ERs.[200] Nevertheless, this interaction partly fulfils ERs ligand requirements and so DDT is a weak estrogen mimic.[201] Indeed, the order of relative estrogenicity to E2, i.e., E2 → BPA → DDT, reflects their structure affinity relationships (Table 3.1).[190, 202-204]

Table 3.1*:* Five molecules that fit ERα's LBC showing their molecular structures aligned to emphasise their molecular analogies, and their relative estrogenicities to E2 (based on MCF-7 cell proliferation studies). [190, 202]

Substance	Structure	Relative estrogenicity to E2
E2		1.0
BPA		1×10^{-3}
Coumestrol		3×10^{-4}
GEN		2.6×10^{-4}

Methylparaben		1.5×10^{-7}
DDT		1×10^{-6}

Molecular modelling studies of the ligand candidates clearly show their different affinities with ERα based on their predicted binding orientation and structural characterises, in particular the spatial arrangements of the key binding groups (e.g., -OH).[205] These complex structure affinity relationships support structure activity relationships leading to different estrogenicities. In addition, the binding between the natural ligand (E2) and ERs (Fig. in1.7) indicate the binding requirements are very similar between ERα and ERβ; however, the biological tests based on ERβ are rare (e.g., the most commonly used test estrogenicity screen (E-Screen) assay, chemical activated luciferase gene expression (CAULX) assay are both ERα-based). In this chapter, an *in silico* system Schrödinger platform to predict flavonoids' binding energies with ERα, and investigate the potential structural effects of flavonoids on their binding affinity and estrogenicity (data from published data).

3.1.6 Overview of docking using the Schrödinger platform and its potential limitations

The docking project describes the use a software package named Glide in Schrödinger platform, this aims to dock molecules from a physical or virtual database (PDB X-ray crystal coordinates) into a receptor derived from a high–resolution crystal structure (e.g., 1ERE

(PDB entry code) of ERα).[206] Glide has been designed to perform as close to an exhaustive search of the positional, orientational and conformational space available to the ligand as is feasible, while retaining sufficient computational speed to screen large libraries.[186, 206] In addition, Glide has been reported to have following performance characteristics: less docking time compared with other docking programs, robustness in binding mode prediction, reasonable binding affinity prediction compared with experimental data for crystallised complexes.[186, 206] Importantly, the scoring functions in Schrödinger have been reported to have great dock accuracy with ERs.[207] However, in the Schrödinger docking studies, because of the utilisation of a rigid receptor (ER) situation, the orientation of amino acid residues are restricted, resulting in limited possibilities of interaction with the docked ligand.[208] This suggests the potential conformational changes caused by different ligands with the LBC cannot be predicated and presented by the docking study, in turn, which may influence the accuracy of the results yielded by docking study. This is the key potential limitation of the docking studies in Schrödinger platform.

3.2 Research objectives

The objectives of the research described in this chapter are:

- Use a computational bio−molecule platform (Schrödinger) to investigate the communication between the LBC and AF-2 of ERα *via* the conformation changes caused by the docked ligand.

- Study the effects of structure properties of ligands (i.e. flavonoids) on their theoretical ERα binding energy, and potential implication for estrogenicity.

3.3 Experiments

3.3.1 Protein preparation for flavonoids docking studies.

The X−ray crystallographic coordinate of ERα were taken from the PDB (http://www.rcsb.org), the X-ray crystal structures of ERα complexed with ligands (e.g., GEN in PDB entry 1X7R) were used. This crystal structure of ERα has common polypeptide chains. Protein fragment crystals are derived from different polypeptides of the ERα protein according to the particular preparative methodologies. This means that they might have different polypeptide chains and each chain has a docked ligand (e.g. GEN). One of these chains was arbitrarily used as the ligand–receptor subunit for these docking studies, others were deleted. Missing amino acid residues (Ser 305, Tyr 331, Asp 332, Pro 333, Thr 334, Arg 335, Pro 336, Phe 337, Arg 548, Leu 549) based on the ERα's primary sequence were added to complete the protein structure using the Schrödinger 'Prime' command. Besides the ligand, a water molecule was kept if it was known to be important for ligand−receptor interaction, [209] others (solvent water) were removed.[209] Restrained Minimization was run to provide controls for optimizing the corrected structure, relieving any strain and fine−tuning the placement of specific groups (e.g., hydroxyls). Hydrogen atoms are always optimized fully, which allows relaxation of the H-bond network and alleviates potential steric clashes by user–selected root–mean–square deviations (RMSD) with a tolerance of 0.3 Å.[186]

3.3.2 Receptor Grid generation

The receptor grid for docking studies was set up and generated from the Receptor Grid Generation panel of Glide (Schrödinger Release 2017-1: Glide, Schrödinger LLC, New York, NY, 2017). The natural LBC was used for these docking studies, but the original bound ligand (e.g., GEN) was excluded; this determines the position and size of the active site (i.e., the LBC) for ligands docking.

3.3.3 Preparation of potential ligands

In this study, 20 flavonoids from different subclasses were select as potential ligands for ERα. They are primuletin, primetin, chrysin, apigenin, norwogonin, acacetin, scutellarein, diosmetin, artocarpetin, 6-Hydroxyluteolin, kaempferol, GEN, glycitein, DAID, FOR, biochaini A, naringenin coumestrol, phloretin. These ligands for the docking studies were built and prepared using LigPrep (Schrödinger Release 2017-1: LigPrep, Schrödinger LLC, New York, NY, 2017); was at most 32 ligand poses were generated.

3.3.4 Ligand docking and calculations of DockingScores

Rigid-receptor-flexible-ligand docking calculations were performed using Glide in extra precision (XP) mode. Each ligand was conformationally sampled in the LBC, and each pose was scored in terms of its Emodel, which is used for selecting the "best" pose (with lowest-Emodel) of a ligand. Glide uses a scoring function–DockingScore to predict the binding energy then evaluates and semiquantitatively ranks the binding affinity of potential ligands with their target receptors in a specified conformation (e.g., 1ERE of ERα).[208, 206] DockingScore is an empirical scoring function; it is used to predict the ligand binding free energy and rank different candidate ligands in order of their binding affinities.[206] DockingScore has many components, including H-bond value, hydrophobic enclosure reward (HER), low molecular weight reward, rotatable bond penalty. In the Glide scoring function of docking study, the optimum distance between two hydrophobic molecules for facilitating hydrophobic interaction is 0.5 Å, but if the distance between ligands atoms and the target atom of an amino acid residues over 3 Å, their hydrophobic interaction will not be accounted.[208] The DockingScores of ligands were used to predict the order of binding affinities of the docked ligands with the receptor. The H-bonds and π−π interaction were shown using a Ligand Interaction Diagram. The hydrophobic enclosure was shown using Corey-Pauling-Koltun (CPK) model. The topographic view of AF-2 was shown in Figure

3.12 using residue property representation: red = negative, blue = positive, green = nonpolar, cyan = polar, grey= neutral.

3.3.5 Protein structure alignment.

Protein structure alignment (Schrödinger Release 2017-1: Protein Structure Alignment, Schrödinger LLC, New York, NY, 2017) was used to investigate the conformational changes triggered by the binding of different ligands at the LBC of ERα. The X-ray crystallographic coordinate of ERα were taken from the PDB, and the protein preparation process is same as descried before (***Section 3.3.1***). Missing amino acid residues (see Table S1, Appendix A) of all the studied complex of ERα with different ligands based on the protein's primary sequence were added to complete the protein structure using the Schrödinger 'Prime' command. The complexes of ERα with ligands were selected and aligned. The alignment score, which describes the structural analogy between any two selected complexes of ERα, was determined. A lower value means the selected two X-ray crystal structures have a greater degree of structural homology.

3.4 Results and Discussion

3.4.1 LBD architecture

As discussed before (***Section 1.2.2, Chapter 1***), in ERα, H3–H12 (Fig. 3.4 (a) are important in the functional architecture of the LBD, which comprises the two binding clefts (i.e. LBC and AF-2). These helices are folded into three−layers, and H5/6, H9 and H10 sandwiched between two additional layers comprising H 1–4 and H7, H8, H11.[54, 20] The LBC comprises segments from H3, H6, H8 and a preceding loop, H11, H12 and the S1/S2 hairpin (Fig. 3.4 (a).[54, 3] This binding site is partitioned from the external environment and occupies a relatively large portion of the LBD's hydrophobic core (Fig. 3.4 (b).[54] The arrangement of these helices creates a 3-dimensional LBC at the narrow end of the LBD with adjacent AF-

2.[54] AF-2 is the hydrophobic cleft formed by the remaining segment of H3 and part of H5, H4 and H12.[54] The sharing of H3 and interlinking of other helices in both clefts suggests the LBC and AF-2 may have architectural contact (Fig. 3.4 (a) facilitating communication between these two binding clefts.

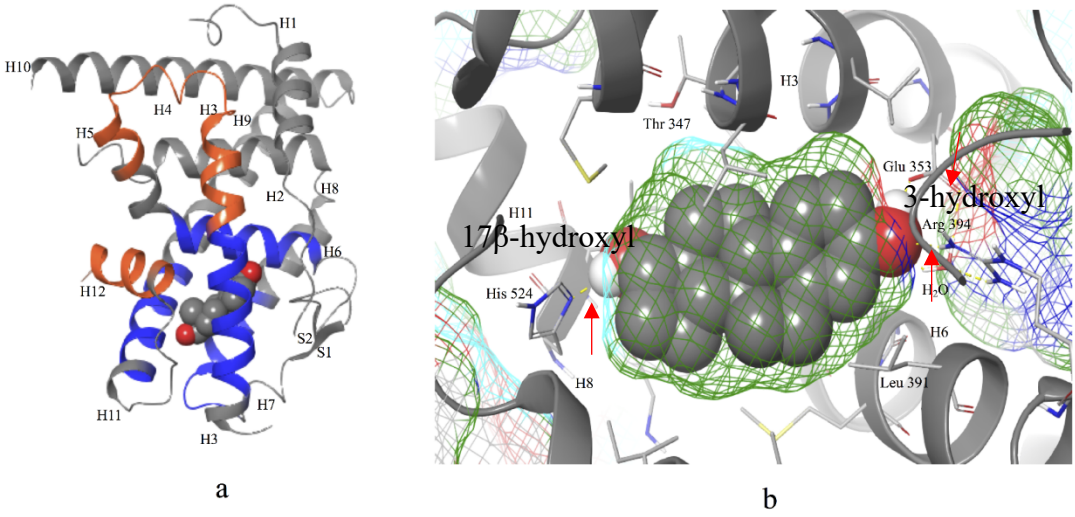


Figure 3.4: Arrangement of helices in the LBD of ERα to create LBC (blue; E2 *in situ*) and H11−linked AF-2 (red−orange) (a). E2 docked in the LBC showing the H-bonds (---, point out with a red arrow) between E2's 3-hydroxy and His-524, and E2's 17β-hydroxyl and the Glu-353, Arg-394 and H_2O triumvirate.[54] Regions of the protein are represented as red for negative, blue for positive, green for nonpolar, cyan for polar, grey for neutral. Amino acid residues interacting with the ligand via H-bonds are shown. All amino acids residues within 3 Å of the ligand are also shown to indicate the chemical nature of the binding pocket. Diagram generated in Schrödinger using the Ligand Interaction Program (See ***Section 3.3.4***).

The arrangement of amino acid resides in ERα's LBC points to ligand specificity; however, the LBC is surprisingly promiscuous being able to accommodate a broad array of ligands providing they have particular molecular attributes.[54] The interaction of the natural estrogen–E2 with ERα, shows the binding requirements; namely, two hydroxyl groups appropriately

separated. These binding requirements can be achieved by various compounds (e.g., flavonoids).[210]

3.4.2 ERα promiscuity and consequences of promiscuity

Interestingly, the total volume of the LBC (450 Å^3) is greater than E2's volume (245 Å^3)[20] which allows for a degree of ligand promiscuity providing the basic tenets of docking are obeyed. [54] These docking requirements are dictated by the spatial arrangement of the key binding amino acid residues (Fig. 3.4 (b);[54] they are ideally an aromatic hydroxyl and an aliphatic hydroxyl with a 9.66 Å separation and a region of hydrophobicity between the docking centres according to E2's structure.[54, 199] However, surprisingly, there is a degree of flexibility about the docking requirements such that electron withdrawing groups (e.g., -Cl) can substitute for hydroxyls, and the strangely large LBC volume means that it can accommodate larger, bulky unnatural ligands (e.g., DDT).[54, 211] More efficient filling of the LBC with a ligand, which has a larger hydrophobic region, might lead to greater engagement between the adjacent hydrophobic amino acid residues and the docked ligand.[54]

The receptor's promiscuity means that a large number of endogenous estrogens (e.g., E3) and exogenous xenoestrogen (e.g., GEN) have been shown to stimulate the proliferation of MCF-7 (ERα–mediated cell line), this suggests these compounds could be recognised and accepted by ERα. [54, 190, 204, 210] Other estrogen mimics could also elicit cellular estrogen responses by the same mechanism. Interestingly, the intimate relationship between ligands and the LBC could be varied because of the different molecular attributes of ligands (e.g., hydroxyl patterns, molecular hydrophobicity), and, this their interactions (i.e., H-bonds and van der Waal's forces) with amino acids residues in the LBC will vary considerably. Alignment of the crystal structures of ERα/ligand complexes shows the comparison of the conformational response by binding with different ligands. For example, aligning the ERα/E2 (PDB entry code: 1ERE) conformation with ERα/methylparaben (PDB entry code: 4TV1) [54, 23] shows a remarkably similar receptor conformational response (Fig. 3.5), even though methylparaben

is significantly smaller than E2 and only interacts with the Glu-353, Arg-394, H_2O triumvirate (Fig. 3.5), which suggests methylparaben has less binding energy and binding affinity than E2 with ERα. [54] This explains methylparaben's estrogenicity (Table 3.1). In addition, an isoflavone, GEN has significant structural similarity to E2 forming H-bonds with same specific amino acids (e.g., His 524); this explains why GEN is accepted as a ligand by the ERα (Fig. 3.6).[209]

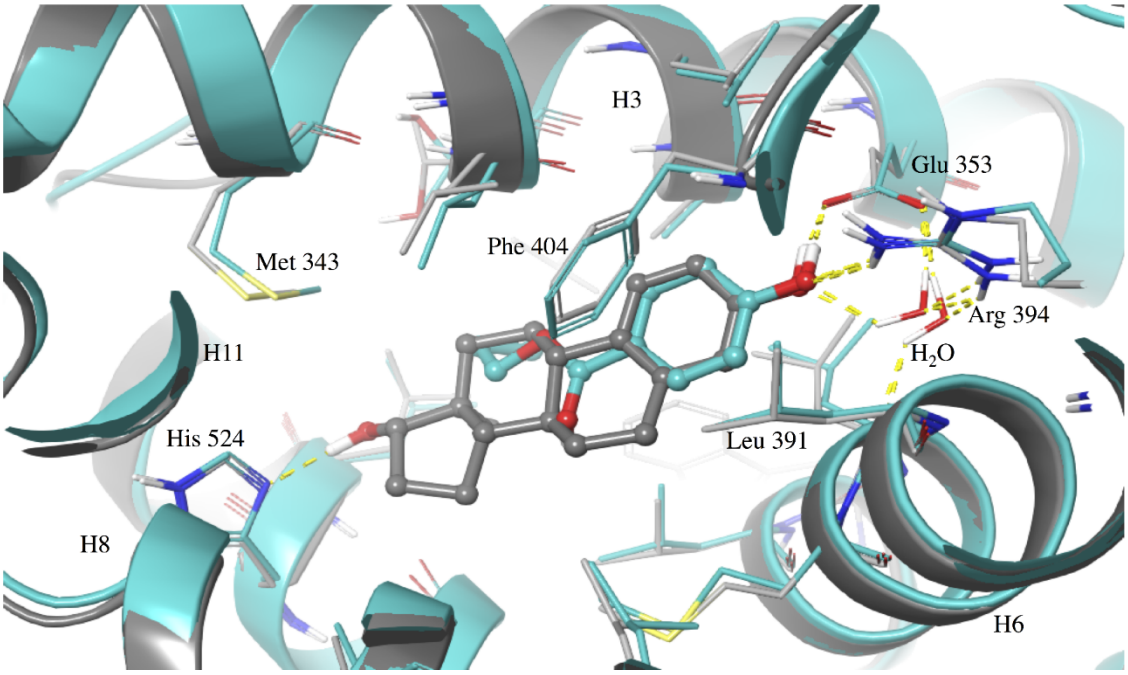


Figure 3.5: Alignment of the crystal structures of ERα complexes with methylparaben (PDB entry code: 4TV1-green) and E2 (1ERE-grey) showing the H-bonds between methylparaben or E2 with LBC. Amino acid residues interacting with the ligand via H-bonds are shown. All amino acids residues within 3 Å of the ligand are also shown to indicate the chemical nature of the binding pocket. Diagram generated in Schrödinger using the Ligand Interaction Program (See ***Section 3.3.4***).

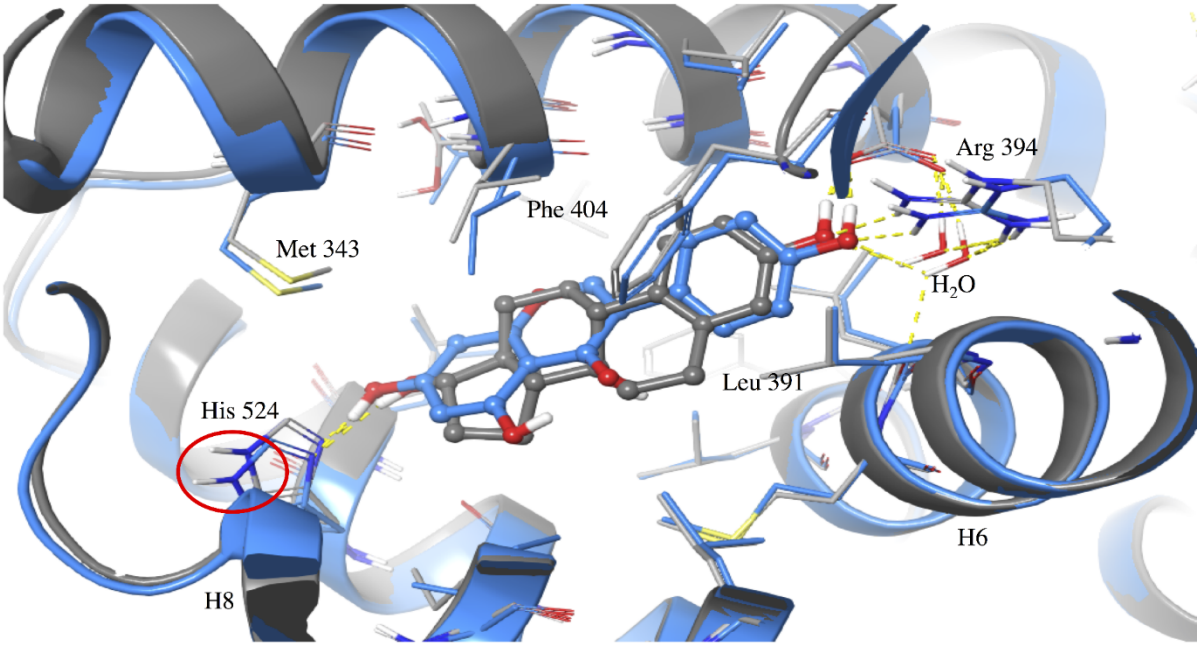


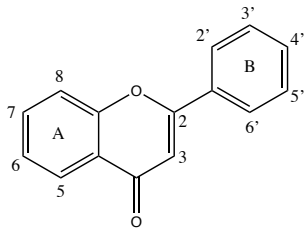
Figure 3.6: Alignment of the crystal structures of ERα complexes with E2 (PDB entry code:1ERE–grey) and GEN (PDB entry code:1X7R–cyan) showing key amino acid residues' orientation and helix positions. The different orientations of His 524 in the both conformations of ERα are shown in a red ellipse. H-bonds are shown as yellow dashed lines. Amino acid residues interacting with the ligand via H-bonds are shown. All amino acids residues within 3 Å of the ligand are also shown to indicate the chemical nature of the binding pocket. Diagram generated in Schrödinger using the Ligand Interaction Program (See ***Section 3.3.4***).

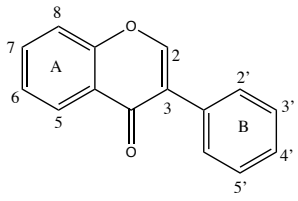
Many flavonoids are phytoestrogens, this means that these compounds likely fit the structural requirements for binding to the LBC of ERα.[8, 92] However, flavonoids have many subfamilies with different molecular skeletons, which lead to different structural features.[212] In addition, within the same flavonoid subfamily; they are different with spatial arrangement of hydroxyl groups. for examples, the isoflavones DAID and GEN have different hydroxyl pattern, which might form different interactions with the LBC resulting in different binding affinities with ERα. This structure diversity will likely influence ligands' bio-potency (i.e., estrogenicity).

3.4.3 Estrogenic activity of food flavonoids and their DockingScores.

Flavonoids are present in many foods (e.g., GEN and DAID in soy) and dietary supplements (e.g., isoflavone supplements for menopausal women); many are estrogen mimics—they comprise part of the complex dietary cocktail that humans are exposed to daily and are possibly responsible for the functionality of these foods.[213] And some flavonoids (e.g., soy isoflavones) are thought to have significant biological effects at the population level (e.g., reduced sperm quality[59]). 20 flavonoids from different subclasses (Table 3.2), representing various structural features, were selected for docking studies in ERα in this chapter.

Table 3.2: Twenty compounds from different subclasses of flavonoids showing different substitution arrangement, DockingScore (kcal/mol), HB value (kcal/mol), and HER (kcal/mol) with the LBC of ERα.

Subclass	Flavonoids	Position & Substituent -	DockingScore (kcal/mol)	H-bond value (kcal/mol)	HER (kcal/ mol)
Flavone & Flavonol	Primuletin	5 (-OH)	−8.13	−1.1	−2.93
	Primetin	5, 8 (-OH)	−8.47	−1.44	−2.82
	Chrysin	5, 7 (-OH)	−8.5	−1.50	−2.7
	Apigenin	5, 7, 4′ (-OH)	−8.64	−1.6	−2.7
	Norwogonin	5, 7, 8 (-OH)	−8.37	−1.44	−2.62
	Acacetin	5, 7, (-OH); 4′ (-OCH_3)	−8.58	−1.1	−2.5
	Scutellarein	5, 6, 7, 4′ (-OH)	−9.04	−2.06	−2.2

	Diosmetin	5, 7, 3′ (-OH); 4′ (-OCH_3)	−8.47	−1.5	−2.4
	Artocarpetin	5, 3′, 4 ′(-OH); 7 (-OCH_3)	−7.56	−2.01	−2.3
	6−Hydroxyl uteolin	5, 6, 7, 3′, 4′(-OH)	−9.37	−3.36	−2.2
	Kaempferol	5, 2, 7, 4′ (-OH)	−8.81	−2.38	−2.2
Isoflavone	GEN	5, 7, 4′(-OH)	−9.3	−1.69	−2.7
	Glycitein	7,4′(-OH); 6 (-OCH_3)	−9.09	−1.6	−2.7
	DAID	7, 4′(-OH)	−9.0	−1.3	−2.8
	FOR	7(-OH); 4′(-OCH_3)	−8.7	−0.78	−2.8

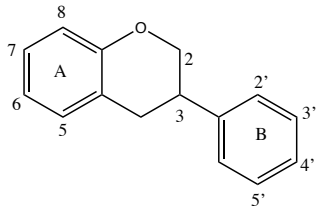
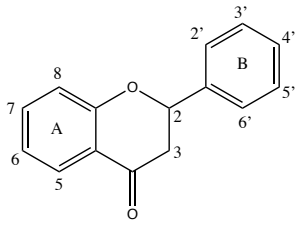
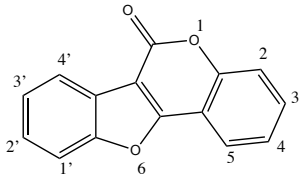
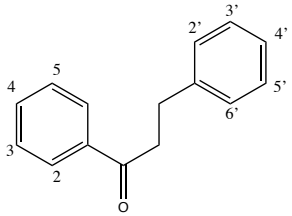
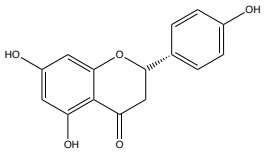
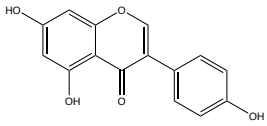
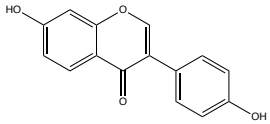
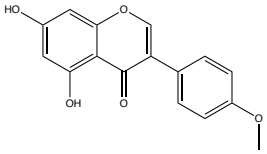
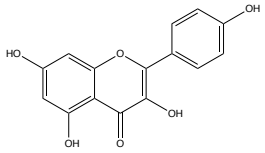
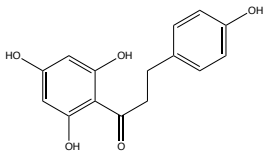
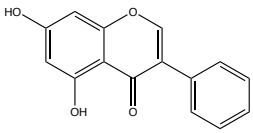
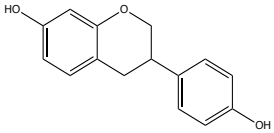
	Biochanin A	5, 7, (-OH); 4′ (-OCH_3)	−8.9	−1.1	−2.7
Isoflavondiol 	*S*-equol	7, 4′ (-OH)	−10.44	−1.3	−2.93
Flavanone 	Naringenin	5, 7, 4′(-OH)	−10.2	−1.44	−2.3
Coumestans 	Coumestrol	3, 2′ (-OH)	−10.4	−1.04	−2.7
Dihydrochalcone 	Phloretin	2, 4, 6′, 4′ (-OH)	−8.3	−2.66	−0.7

Table 3.2 Subclasses of flavonoids - the different subclasses have different molecular skeletons which maybe the hydrophobic cores of ligands for ERs, and might form important hydrophobic interactions with ERα. Within each subclass, compounds have different substitution arrangement (i.e., hydroxyl groups and methoxyl groups). These structural characteristics contribute to different H-bond values, HERs, thus give different DockingScores shown in Table 3.2.

Over 5,000 naturally occurring flavonoids have been identified in plants;[214] however, surprisingly few of them have been studied using functional assays to determine, for example, their estrogenicity.[215] Table 3.3 shows published relative estrogenicity (determined by yeast estrogen screen assay (YES assay), [190] for the flavonoids studied in this chapter (estrogenicity values are not available for all the flavonoids studied), where E2's value equals 1, the DockingScores of selected flavonoids obtained from the Docking study in this chapter. The molecular structures show that these flavonoids have the key common structural features: namely, hydroxyl groups and a hydrophobic backbone, this means they have similar structural and spatial arrangements of the key functional groups (i.e., hydroxyl) to E2's key moieties. These two important structural features might facilitate interactions between these candidate ligands and the LBC of ERα by H-bond formation and hydrophobic interactions. Interestingly, the order of estrogenicities of the compounds studied is the same as the order of their DockingScores (Table 3.3). This trend indicates a potential association between the ligand/receptor docking energy (i.e., DockingScore) and estrogenicity.[190, 216]

Table 3.3: Food sources, structure, estrogenicity and DockingScores with ERα of selected flavonoids.

Compound	Examples of food source	Structure	Relative estrogenic activity[190]	DockingScore (kcal/mol)
Naringenin	Grapefruits, oranges		7.8×10^{-3}	−10.2
GEN	Fava beans, soybeans		4.5×10^{-3}	−9.3
DAID	Soybeans		2.8×10^{-4}	−9
Biochanin A	Peanuts, chickpeas, soybeans		2.5×10^{-4}	−8.9
Kaempferol	Apples, potatoes, blackberries		1.1×10^{-4}	−8.81
Phloretin	Apples and pears		9.4×10^{-3}	−8.3

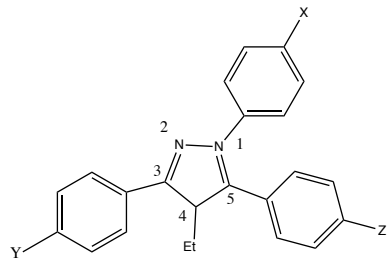
Chrysin	Oyster mushrooms		2.5×10^{-4}	−8.5
***S*-equol**	Gut microbiome metabolite from DAID		2.3×10^{-2}	−10.44

3.4.4 Does the H-bond value determine a ligand's binding affinity to ERα?

The H-bond value is the sum of the individual H-bond energies based on both the angles and distance between the donor and acceptor atoms in the bond. It is determined by the relative orientation and distribution of the bound atoms of ligands and the corresponding interacted receptor atoms.[217] This means that the H-bond value could be affected by both distribution and orientation of the ligand's hydroxyls which might form H-bond with amino acid residues in the LBC.[217] There are two interesting trends in the data shown in Table 3.2. Firstly, the ligands, which have more phenolic hydroxyls, have larger H-bond values. This is likely due to the increased number of H-bonds formed between the ligand's -OH groups and the amino acid residues at the LBC. In addition, this trend is exemplified by the following flavones which have different numbers of hydroxyls and different H-bond values—primuletin (monohydroxy, −1.1 kcal/mol), primetin (dihydroxy, −1.4 kcal/mol), apigenin (trihydroxy, −1.6 kcal/mol) and two isoflavones, DAID (dihydroxy, −1.3 kcal/mol) and GEN (trihydroxy, −1.69 kcal/mol). In addition, GEN has higher estrogenic activity than DAID (Table 3.3), this suggests H-bond might influence the ligand's estrogenicity. This is logical since the more hydroxyl–mediated H-bonds between the ligand and its receptor, the stronger the interaction resulting in higher binding energy and binding affinity, and thus resulting in the greater the receptor–mediated biological response (e.g., estrogenicity).

The understanding of H-bond interaction in the ligand–receptor binary system has been supported by the previous study of pyrazole ligands interacted with ERα.[197] This study shows triphenol pyrazole ligands have greater ERα relative binding affinity (RBA) than monohydroxy and dihydroxyhenols (where the binding affinity E2 equals 1) (Table 3.4).[197] This might be due to the additional hydroxyls of triphenol ligands can form more H-bond resulting in higher binding energy, and thus lead to higher RBA compared with monohydroxyl- and dihydroxyl-phenol pyrazole.[197] The effect of H-bond interaction has been also proved by the previous research of an enzyme/inhibitor system (i.e., xanthine oxidase (XO)/flavonoid).[218] The research shows that, apigenin has one more hydroxyl group than chrysin (Table 3.2), the additional hydroxyl of apigenin might form additional H-bond with XO, which would increase the binding energy of apigenin and binding affinity with XO. In addition, the published date shows apigenin has a greater binding affinity and stronger inhibitory activity than chrysin with XO.[218]

Table 3.4: RBA of 4-ethylpyrazole tri-, di-, and monophenol, where the binding affinity of E2 equals 100%*.[197] Et = ethyl; X, Y, Z are three substituent positions.



X	Y	Z	RBA with ERα
-OH	H	H	3.1
-OH	-OH	H	8.9
-OH	-OH	-OH	36

Six of these flavonoids with different polar substitution arrangement (i.e. hydroxy and methoxy) were selected to investigate more details of the H-bond interactions with the LBC of ERα. Figure 3.7 illustrates a trend that the amount hydroxyls of ligand influence the amount hydrogen bonds between the ligand and the LBC. For example, primuletin

(monohydroxyl) forms one hydrogen (Fig. 3.7 (a), norwogonin (trihydroxyl) forms two hydrogen bonds (Fig. 3.7 (b), scutellarein (tetrahydroxyl) forms three (Fig. 3.7 (c), and 6-hydroxy luteolind forms four hydrogen bonds with the LBC (Fig. 3.7 (d). In addition, Figure 3.7 also shows that methylation of vicinal dihydroxy flavonoids (e.g., Biochanin A) changes ligand interactions with ERα; this likely results from that methylation sterically hinders the ligands interaction with the receptor, forcing the unmethylated hydroxyl to interact with a different amino acid residue (Fig. 3.7 (e) and (f), and resulting in a lower binding affinity with ERα.[219] This finding may explain why GEN is more estrogenic than Biochanin A (Table 3.3).[220] An extra polar substituent might interfere with ligand ERα hydrophobic interactions;[221] this likely results from the extra polar substituent causing unfavourable interactions at the hydrophobic region of the LBC and influencing the binding energy and binding affinity of the ligand; for example, kaempferol has one more hydroxyl than GEN and showed lower estrogenicity in the previous research of YES assay (Table 3.3). In summary, the potential effects of polar substitutions on ligand binding affinity and estrogenicity depends on their substituents positions and orientations.

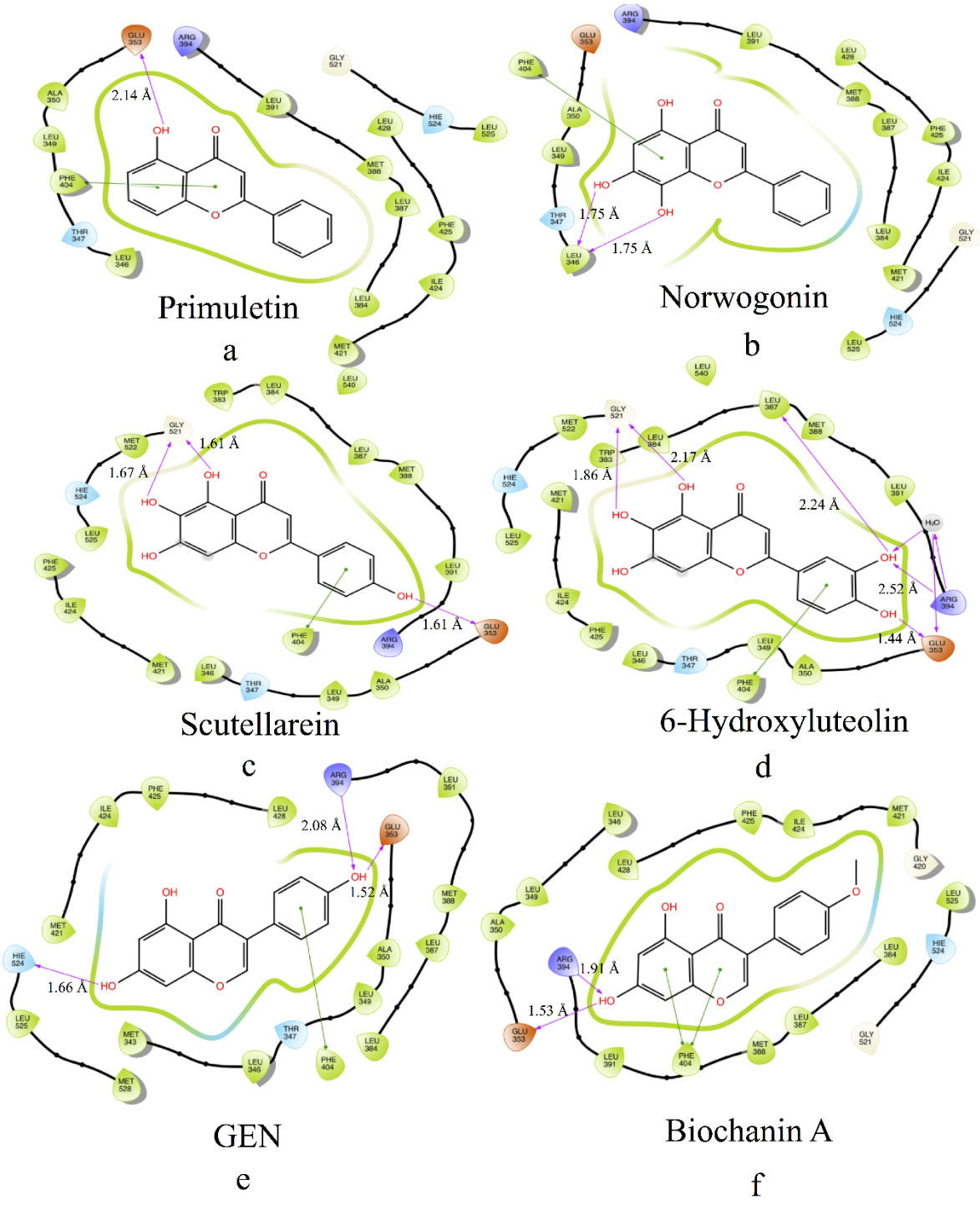


Figure 3.7: Ligand interactions with amino acid residues in ERα visualised in Schrödinger. Purple arrows represent H-bonds, direction of arrow denotes donor to acceptor in the H-bond. Different coloured residues represent amino acid charge properties: blue = positive charge, red = negative charge, cyan = polar, green = nonpolar.

3.4.5 Does the HER determine a ligand's binding affinity with ERα?

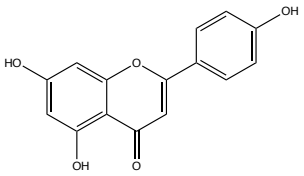
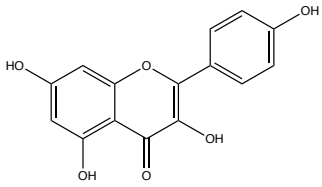
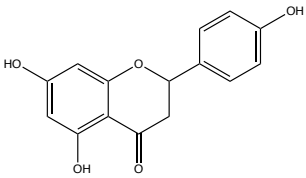
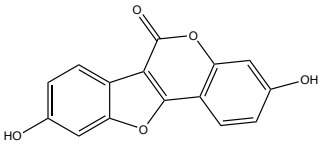
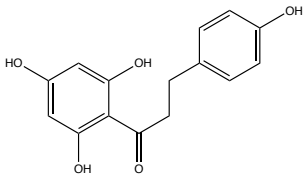
The HER is the term used to differentiate the relationship between the hydrophobicity of ligands with the geometric receptor environment.[208] This describes the spatial arrangement of hydrophobic amino acid residues (e.g., Leu) in a ligand binding cleft of[217] for example, a receptor (e.g., ERα) which supports hydrophobic interactions between a ligand and the receptor;[217] I term this a parallel arrangement. For the perfect interaction, a hydrophobic ligand should be enclosed in a hydrophobic microenvironment in the LBC to give the maximum potential for hydrophobic interactions—ideally this would involve the ligand sandwiched between two hydrophobic regions of the LBC.[208] Previous research indicates that, thermodynamically a hydrophobic ligand 'prefers' to sit in a hydrophobic binding cleft comprising hydrophobic amino acid residues (e.g., Leu and Val);[206, 222] conversely, increased hydrophilic character reduces ideal thermodynamic fit to a hydrophobic cleft.[217] The balance between hydrophobicity of a ligand to hydrophilic character of the accommodated ligand determines its binding energy and binding affinity with the receptor.[217]

Six compounds from different subclasses of flavonoids were selected for study the contribution of molecular skeletons to the ligands' theoretical binding energy (i.e., DockingScore) of the selected ligands with ERα. As can be seen from Table 3.5 and Figure 3.8, coumestrol has the highest HER, this is likely due to coumestrol binds to the LBC and sits 'parallel' to the binding cleft's hydrophobic region. Phloretin has more (i.e., 2) rotatable bonds (Fig. 3.9) than apigenin, kaempferol and naringenin (i.e., 1), while coumestrol has no rotatable bonds (Fig. 3.9) resulting in the lowest HER (Table 3.5). This trend indicates, in the same binding environment particularly having a same hydrophobic region, that a ligand sits parallelly could bind more strongly than a ligand with a non−parallel molecular arrangement (i.e., with polar substitution outside the parallel plane; for example, kaempferol (Table 3.5).

The impact of rotatable skeleton of ligands on their binding affinity and bioactivity is also supported by a previous study of an enzyme/inhibitor system (i.e., XO/flavonoid), which

requires structural requirements of ligands for their inhibitory activity.[218] The previous study suggests that, the flavonoid's planar structure and the presence of a non-rotatable C2=C3 (Table 3.2) bond could lead the ligand to sit parallel in the binding domain of XO and this contribute to this ligand's binding affinity, thus promoting its inhibition of the enzyme; for example, the comparative between naringenin and apigenin (Molecular structures are shown in Table 3.2).[218]

Table 3.5: Examples of flavonoid subclasses and their hydrophobic enclosure reward.

Subclass	Example compound	HER (kcal/mol)
Flavone	Apigenin 	−2.7
Flavonol	Kaempferol 	−2.3
Flavanone	Naringenin 	−2.3
Coumestan	Coumestrol 	−2.7
Dihydrochalcone	Phloretin 	−0.7

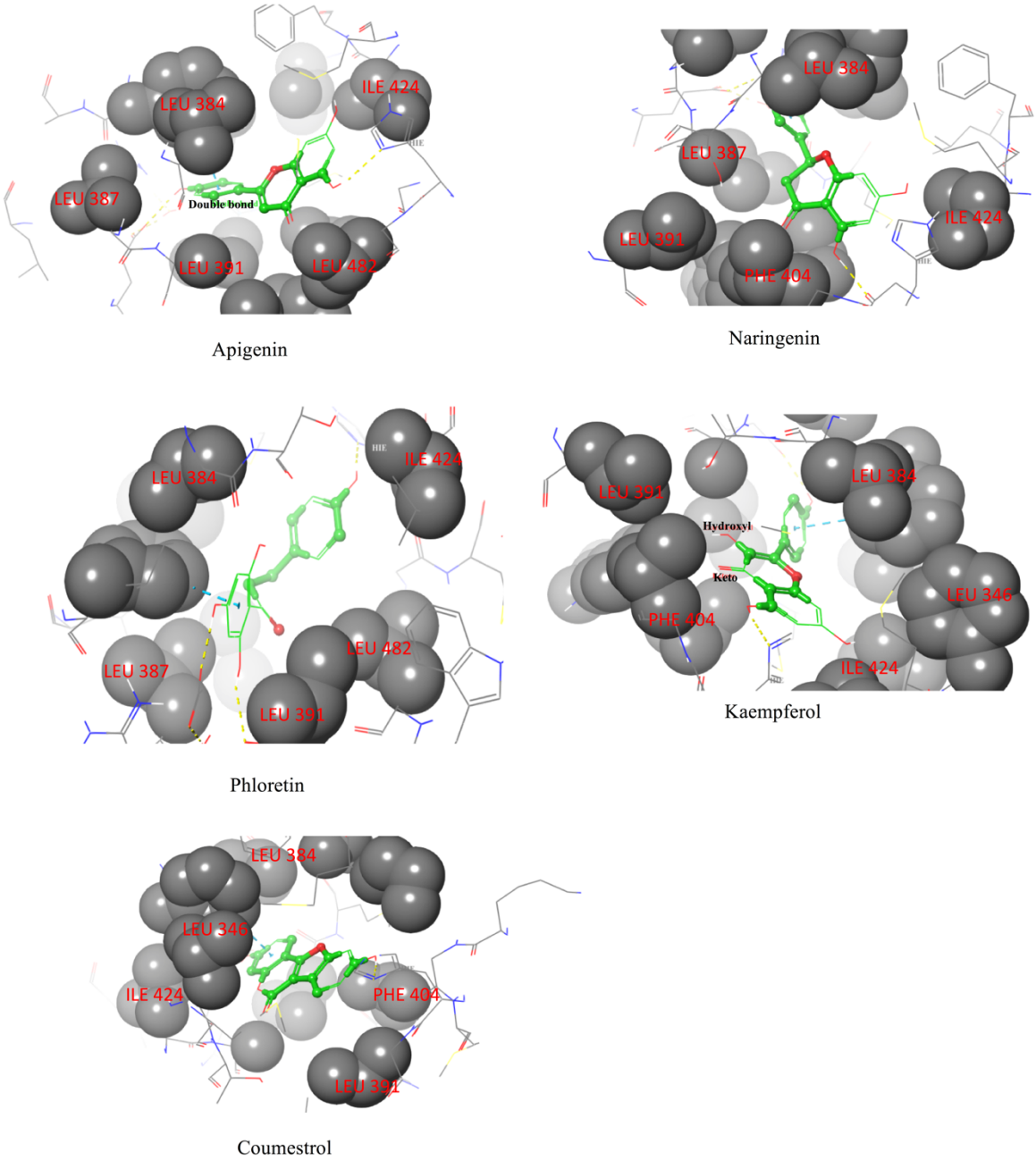
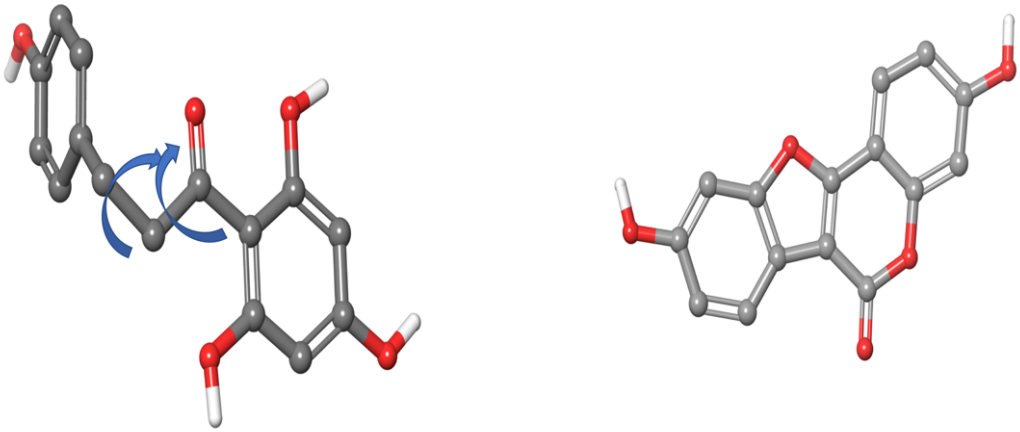


Figure 3.8: Selected flavonoids and their hydrophobic enclosures in the ERα LBC; the ligand (green) is shown by ball and stick representation. Hydrophobic amino acid resides are labelled within 3 Å of the ligand are also shown as CPK representation.



Phloretin Coumestrol

Figure 3.9: Molecular structures of phloretin and coumestrol showing rotatable bonds in phloretin, but there are no rotatable bonds in coumestrol

3.4.6 Gut biotransformation of flavonoids might produce metabolites with different estrogenicities than their parent flavonoids.

Interestingly, gut microbiome−mediated biotransformation of some flavonoids results in the formation of metabolites with different estrogenicities to their parent compounds; for example, DAID is metabolised to the more estrogenic *S*-equol (Table 3.3, Fig. 3.3),[190] and dihydrogenistein, which is a gut−mediated metabolite of GEN, is less estrogenic than its parent compound (Fig. 3.10).[223] The different estrogenicities of metabolites compared with their parent compounds likely result from their different structural features. For example, *S*−equol does not have a keto group (Fig. 3.3), which was predicted to have a greater HER than DAID and thus resulting in higher binding affinity (Table 3.3). This keto group might be the key factor that differentiates the estrogenicities of *S*−equol and DAID. In addition, dihydrogenistein does not have a double bond on the pyran ring whereas GEN does, this means that the molecular structure of dihydrogenistein is more rotatable than GEN (Fig. 3.10), this could result in dihydrogenistein having a lower hydrophobicity and thus a lower binding affinity with ERα than GEN. The change of estrogenicity during flavonoid's metabolism might result in changes in bioactivity which, in turn, could lead to both health

benefits and risks. For example, increasing estrogenic load could be beneficial in peri−menopausal women who are responding physiologically to the natural reduction in their estrogen levels as the menopause progresses. Dietary flavonoids, or flavonoid dietary supplements, might ameliorate symptoms of menopause,[224] especially if microbiome−mediated biotransformation of the flavonoids leads to increased estrogenicity. However, on the risk side, men exposed to dietary flavonoids might develop ERα−mediated adverse effects at a population level, including reduced sperm count[225] and gynecomastia.[63] Similarly, pre-pubertal girls (they have low estrogen levels[226]) might be triggered to enter puberty earlier by dietary flavonoid exposure because female puberty requires an estrogen stimulus.[227] This will likely be exacerbated if microbiome-mediated biotransformation increases the dietary flavonoids' estrogenicities. Indeed, it is thought that increased exposure to environmental estrogen mimics is related to an increased incidence of precocious puberty in girls worldwide.[227]

The quantitative production of *S*-equol from DAID differs between individuals, this possibly reflects their gut microbiome composition because it is likely that not all bacterial species convert DAID to *S*-equol.[228] This points to the possibility that inter-individual variation in gut microbiome composition might influence the impact of dietary flavonoids on human health.[229]

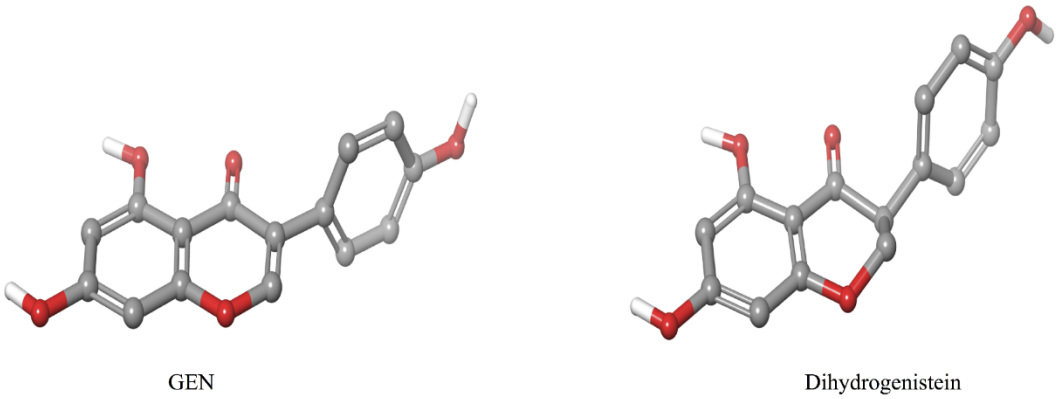


Figure 3.10: Three-dimensional representation of the structures of GEN and dihydrogenistein showing the absence of a carbon double bond in dihydrogenistein allowing distortion of the ring resulting in a more rotatable structure compared with GEN.

The *in silico* study of flavonoids reported here indicates these ERα ligands could have different interactions with the LBC because of their structural differences. Interestingly, in the protein alignment study, the X-ray crystal structure of ERα complexed with GEN (PDB entry code: 1X7R) shows a slight shift at His-524 on H11 compared with E2's (PDB entry code: 1ERE) (Fig. 3.6), which implies a difference in the conformational response of ERα to these ligands. In addition, as discussed above (***Section 3.4.1***), the LBC and AF-2 shares H12, and H12 is linked to H11 *via* a polypeptide loop. This means, a ligand might affect the conformation of AF-2 by interacting with H11 (Fig. 3.2), or, of course, direct interaction with H12 may lead to the AF-2's reconformation. [230]

3.4.7 The LBC's plasticity

Protein alignment of different complexes of ERα/ligand in this study shows that he docked ligands with bulky side substitutions have greater effects on the conformation of the LBC with concomitantly greater communication effects on AF-2 compared with the natural ligand, E2. This can be visualised by aligning structures derived from X-ray crystallography of the ERα's LBC with E2 (PDB entry code: 1ERE) and an agonist model compound, oxabicyclic heptene sulfonate (OBHS) (PDB entry code: 5U2D[54, 176]), the alignment score is 0.016

(Table 3.6). This protein alignment shows that H11 responds explosively to OBHS and moves some 90° compared to H11 with E2 docked; this is illustrated by the relative positions of the Lys-531 residue (Fig. 3.11). The knock-on effect on AF-2 caused by the docked OBHS is mediated by the polypeptide loop connecting H11 and H12, this results in a significant change in the conformation of AF-2. For example, Lys-531 on the H11 and Glu-542 on the H12 significantly change their relative spatial arrangements (Fig. 3.11). Indeed, raloxifene caused greater knock-on effect on the AF-2 facilitating antagonist conformation of the LBC, and resulting in higher alignment score (0.044) compared to other ligand/ERα complexes (e.g., GEN/ERα and OBHS/ERα) (Table 3.6).

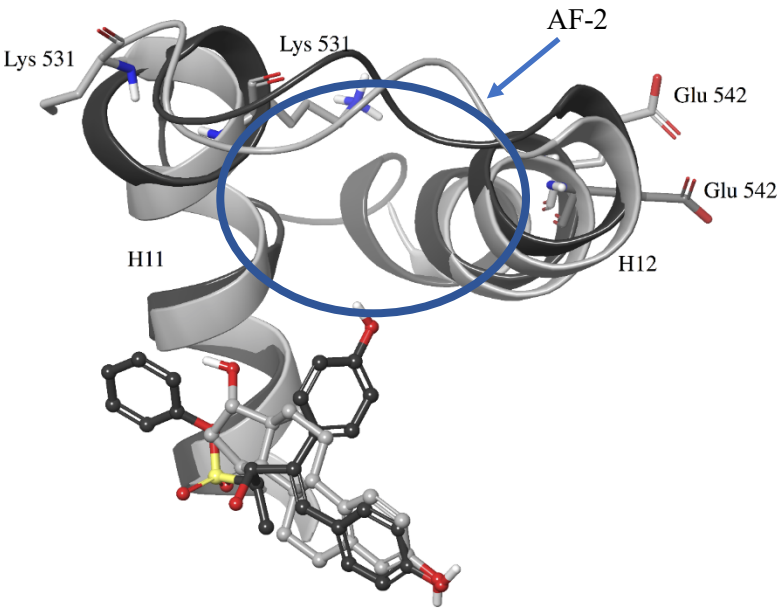
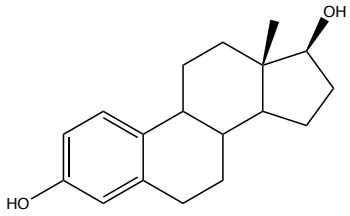
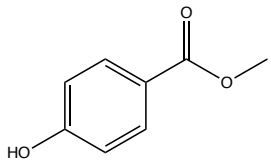
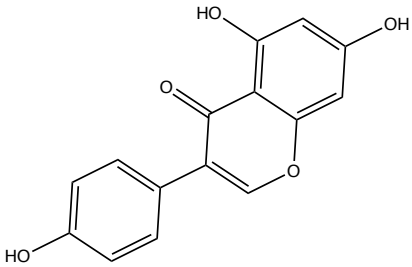
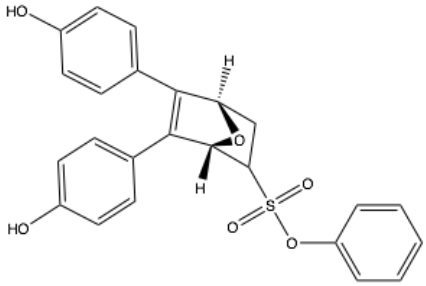
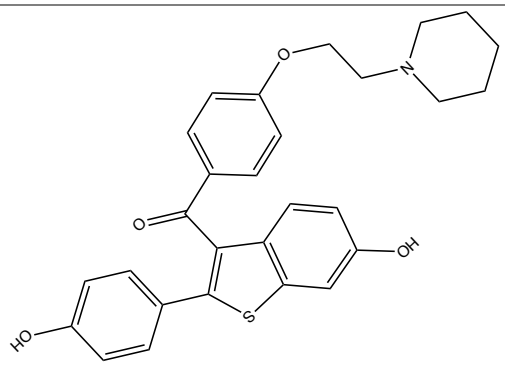
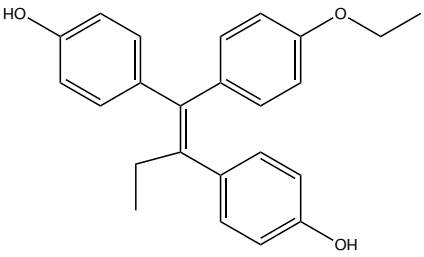


Figure 3.11: Crystal structures of the complexes of ERα with E2 *in situ* (1ERE–grey) aligned with ERα with OBHS *in situ* (5U2D–black) showing spatial arrangements of key helices—the position of H11 differs greatly between the two crystals which would have significant knock-on effects to AF-2. AF-2 is shown in a blue ellipse. Helices are represented by ribbons.

Table 3.6: Alignment scores between different ligand/ERα complexes.

Substance	Structure	PDB entry code	Alignment score
E2		1ERE	Not Applicable
Methyparaben		4TV1	0.012
GEN		1X7R	0.009

OBHS		5U2D	0.016
Raloxifene		1ERR	0.044
Ethoxytriphenylethene (ETP)		5T1Z	0.014

3.4.8 ERα LBC/AF-2 topography.

A topographic view of the outer surface of the LBD reveals the binding environment presented to the regulatory protein.[54, 23] The amino acid residue properties presented to approaching prospective ligands are very different with E2 docked compared to the docked bulky OBHS (Fig. 3.12). For example, with E2 docked (PDB entry code: 1ERE), the surface of AF-2 shows Glu-542 exposed whereas OBHS-bound ERα shows both Glu-542 and Glu-380 exposed (Fig. 3.10). This presents a more negative (red in Fig. 3.12) electrostatic

environment to an approaching regulatory protein and likely influences the interaction between the regulatory protein and AF-2. Further to this, the topographical charge distribution is quite different between these two ligand/ERα complexes (Fig. 3.12) which again alters the interaction potential between regulatory proteins and the AF-2 binding cleft.

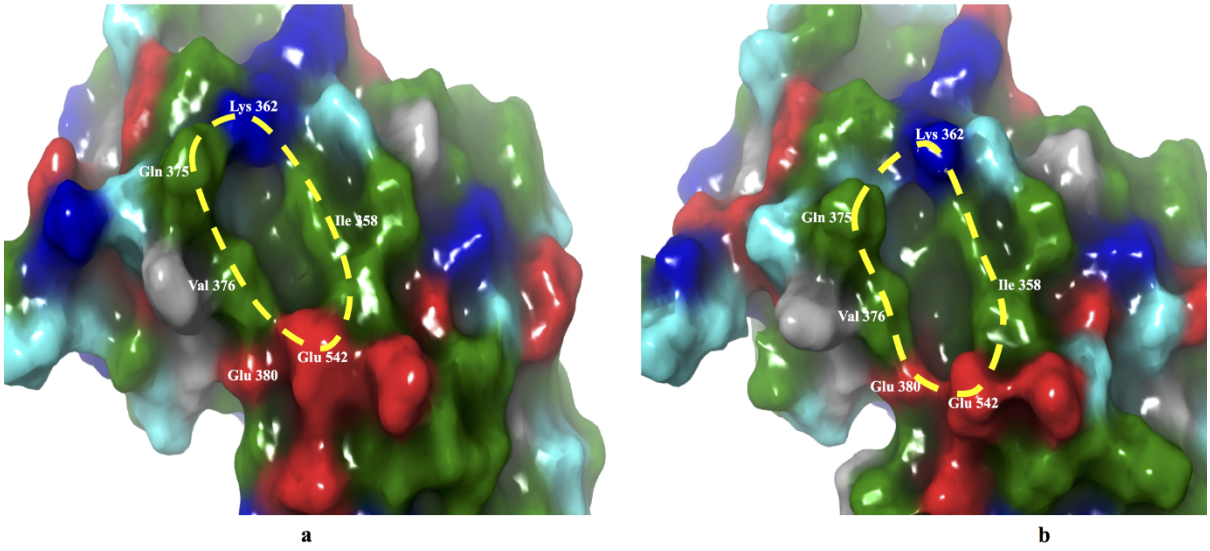


Figure 3.12: Topographic views of the AF-2 of ERα bound with E2 (a) and OBHS (b). Regions of the protein are represented as red for negative, blue for positive, green for nonpolar, cyan for polar, grey for neutral. Orange dashed lines show the shape of AF-2.

Aligning published ERα crystal structures with different ligands *in situ* gives an invaluable insight into the effects of ligand structures on the different conformations of LDB. The implications of these conformational changes for cellular outcomes are at present very difficult to determine, but it is clear that the molecular structures of ERα's ligands have an enormous effect on the helix pattern of the LBC and AF-2 knock-on conformational changes. The bulkiness of ligand side substitutions is perhaps the best determinant of conformational change and thus is a key factor in designing both agonists and antagonists for the LBC which likely have flow-on effects to AF-2; such agonists might be useful in the treatment of

menopausal symptoms, and antagonists might be apply for the treatment ERα positive breast cancer.

In addition, the alignment of the LBD's crystal structure with E2 docked ERα complex and bulky alien ligands (e.g., OBHS) *in situ* shows a significant bulkiness effect in initiating receptor conformational changes. This conformational change facilitates different spatial arrangements of amino acid residue in the AF-2; which might lead to might form different interactions with the regulatory protein. For example, an ERα/E2 LBD crystal (PDB entry code: 1ERE) aligned with ERα LBD crystals with the following docked ligands *in situ*: the breast cancer drug, raloxifene (PDB entry code: 1ERR[20]), ETP (5T1Z [54, 231]) (Table 3.6) or GEN (PDB entry code: 1X7R) provides insight into intimate and subtle ligand/receptor communications.

The effect of raloxifene on the position of H12 (purple in Fig. 3.13) is profound when compared to ETP's (orange in Fig. 3.13) and GEN's (cyan in Fig. 3.13) effects; this is probably due to raloxifene's ethylpyridine side group which literally pushes H12 away and cause the rearrangement of AF-2.[54,178] E2, GEN and ETP do not have correspondingly bulky groups like raloxifene (Table 3.6). Indeed, H12 is affected similarly by E2, GEN and ETP, which is very different to the H12's explosive effect caused by the docked raloxifene (Fig. 3.13). These more 'conventional' facilitate the formation by conformational changes of the AF-2 which can accommodate the regulatory protein. These differences distinguish agonists from antagonists.[54, 20]

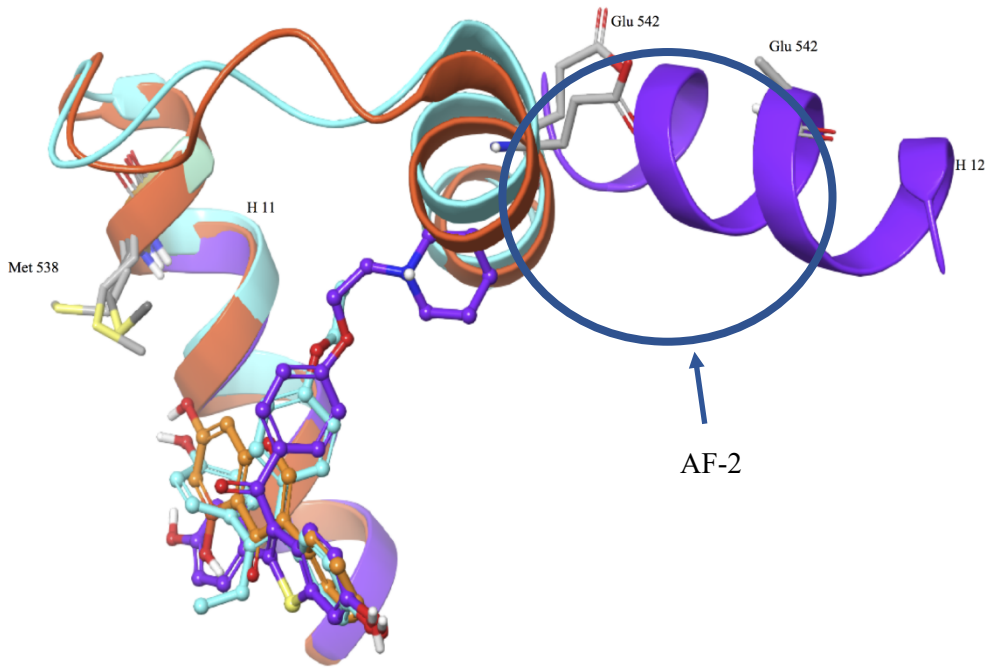


Figure 3.13: ERα with Raloxifene *in situ* (1ERR–purple) aligned with ETP (5T1Z–orange), and GEN (1X7R–cyan) *in situ* showing the spatial arrangement difference of key helices − the position of H12 differs greatly in the Raloxifene crystal compared to the others. Minor orientation differences in H12 for ETP (5T1Z–orange) and GEN (1X7R–cyan) *in situ* in ERα are illustrated by the differences in the spatial arrangements of Asp-538 and Glu-542. AF-2 is shown in a blue ellipse. Helices are represented by ribbons.

A comparison between ETP and GEN *in situ* in the LBC illustrates that GEN results in a conformational change which facilitates H-bonding of the GRIP-peptide (a fragment of a regulatory protein) *via* Glu-542 in AF-2; whereas, ETP bound to the LBC causes a different conformational change which does not favour H-bond formation (Fig. 3.14). This is likely because Glu-542 on H12 is re-orientated when ETP is bound compared GEN is bound, and might resulting in different binding affinities. Occupancy of AF-2 by a regulatory protein is important in modulating ERα's biological activity and so the conformational differences resulting from GEN's or ETP's occupancy could alter ERα's response—this might explain differential estrogenicity.[54, 232]

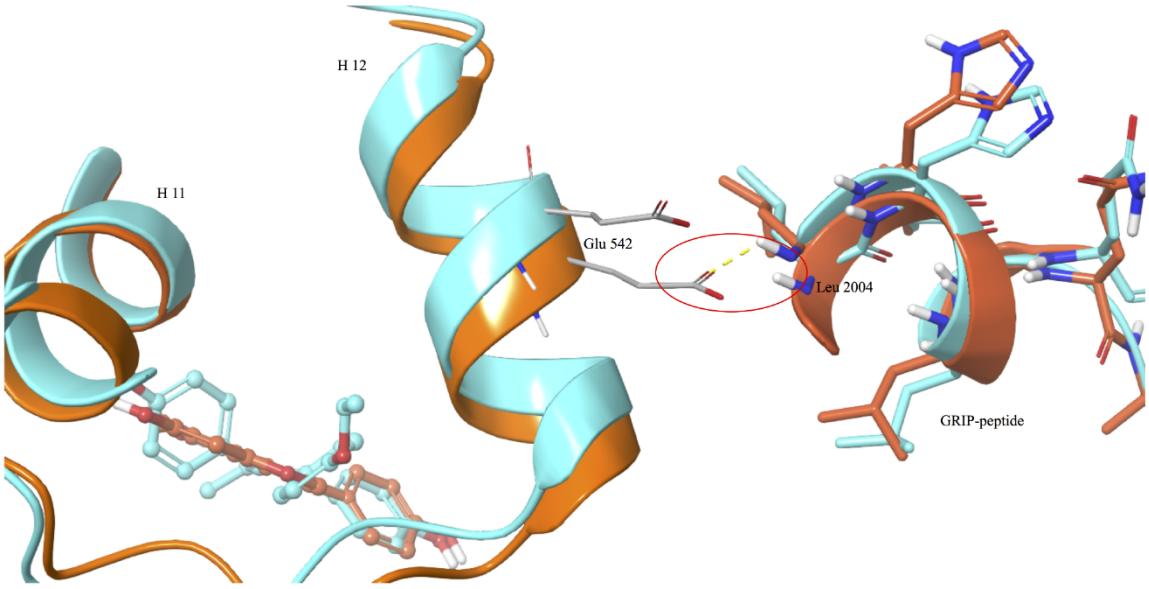


Figure 3.14: AF-2 of ERα with ETP *in situ* (orange–5T1Z) aligned with AF-2 of ERα with GEN *in situ* (cyan–1X7R) with regulatory protein fragment (GRIP-peptide) bound to AF-2 (orange or cyan according to the crystal structure from which it was derived). This shows that the regulatory protein fragment bound to ERα/GEN H-bonds to Glu-542; whereas, the regulatory protein bound to ERα/ETP has no corresponding H-bond. H-bonds are shown as yellow dashed lines. Helices are represented by ribbons.

3.4.9 Regulatory proteins

Regulatory proteins are DNA-binding transcriptional regulators, which are recruited to the AF-2 when the ERα /ligand dimer binds to the ERE.[54, 232] There are two types of regulators, coactivators and corepressors, which up- or down-regulate, respectively, ERα-mediated transcription.[54, 16] Coactivators are histone acetyltransferases-acetylation loosens the chromatin structure, exposing the target genes to the transcriptional machinery.[54, 16] Recruitment of coactivators influences this process, leading to gene transcriptional changes which might underlie differential estrogenicity.[54, 16]

Previous study shows a fragment (SRC-1a) of a regulatory protein has clear differences in affinity for AF-2 depending on the ligand bound to the LBC of ERα (Table 3.7).[54, 233] In Table 3.7, Relative Recruitment Ability (RRA) of AF-2 for SRC-1a with GEN docked in the LBC is 0.06, and for BPA is 0.0003 (assigning 100 to E2).[54, 233] This is likely due to the different binding affinities of the regulatory protein with AF-2. When different ligands bind to ERα they result in different conformational changes in the LBC causing various knock-on effects on the conformation of AF-2. These modified conformations and binding potentials of AF-2 present a different amino acid residue orientation which leads to diverse binding environments for regulatory proteins. This means that the binding affinities of regulatory proteins to different ligand/ERα complexes are likely to lead to differential bioactivities. *In vivo*, this might be extrapolated when a complete regulatory protein is involved, the LBC ligand might alter the nature of regulatory protein binding to AF-2 which would have significant implications for estrogenic activity *via* a gene regulatory mechanism.

Table 3.7: Relative recruitment ability (RRA) of different ligand/ER complexes with fragments (TIF2 and SRC−1a) from different regulatory proteins.

Ligand	RRA TIF2	RRA SRC−1a
E2	100	100
DES	11	5.0
GEN	0.005	0.06
BPA	< 0.0001	0.0003

In summary, a ligand that binds to the LBC could determine bioactivity *via* the conformational change induced in the AF-2, which, in turn, modulates regulatory protein recruitment. The strength of interaction between regulatory proteins and AF-2 is likely to be important for the bioactivity of a ligand bound to ERα.

3.5 Conclusions

This chapter has three major outcomes. Firstly, it shows the LBD's architecture of ERα and the potential communication between the LBC and AF-2 *via* the sharing of H3 and H12. In addition, the knock-on effects of LBC docked ligands on H11 in the LBC could trigger a further conformational change to H12 resulting in reorientation of exposed amino acid residues, facilitating in a topographic change which exposed AF-2.

Secondly, with a good understanding of the ligand binding environment of the LBC and ligand binding requirements, the *in silico* modelling study of flavonoids with ERα (PDB entry code: 1X7R) demonstrated that, theoretically, the structural features of different ligands (e.g., hydroxyl groups, degree of hydrophobicity) can affect noncovalent interactions (e.g., H-bond interaction and van der Waals' interaction) between ligands and the amino acid residues in the LBC; this results in different binding affinities, which could, in turn determine the estrogenicity of ligands and the functionality of, for example, phytoestrogen-containing foods. A detailed understanding of the interaction of ligands with ERα gives insight into their potential biological activity; this could be in a pharmacological or a functional food setting, and so might play a role in designing pharmacologically active ligands (e.g., in the treatment of ER positive breast cancer). In addition, the understanding of the functionality of flavonoid−containing foods might be important in a human health context. *In silico* modelling studies might help to predict functionality and potential bioactivity of food components which could form the basis of tailoring food consumption to personal health needs.

Thirdly, this chapter shows that an investigation of the chemical biology of the intimate interactions between the LBC and its ligands; this provides a molecular basis for understanding different levels of ERα's biological activities. Individual ligands result in varying conformational changes in the LBC, which, in turn, lead to different knock-on effects on AF-2 *via* the sharing architectural components (e.g., H12) with the LBC. The different knock-on effects determine orientations of amino acid residues presenting different charge environments for hosting regulatory proteins as they approach the AF-2. This means the time of bindings between a regulatory protein and the ligand/ ERα complexes might be different. The length of time that regulatory protein−bound ERα sits on the ERE determines the gene product output and therefore results in different biological activity.

Chapter 4: Studies on structure interaction relationships of isoflavones with ERα: *In silico* and in a gene reporter bioassay (MELN)

4 Chapter 4 – Studies on structure interaction relationship of isoflavones with ERα: *In silico* and in a gene reporter bioassay (MELN)

4.1 Introduction

4.1.1 The interactions between the LBC and their ligands

As discussed before (***Section 3.4.1, Chapter 3***), the structure of the natural ligand, E2 and its interactions with the LBC indicates that the ideal binding characteristics for ERα are two hydroxyl groups in the separated by a hydrophobic skeleton. The *in silico* study in ***Chapter 3*** indicates that both amount and orientation of hydroxyl groups are important in the interactions between the ligand and the LBC, this may determine the binding energy, thus influence receptor-driven activity (i.e., estrogenicity). The importance of the different orientations of hydroxyl group can be also proved by the overlay of 17α-estradiol and E2 (Fig. 4.1); this influences the presence of H-bond between the ligand and the LBC, which, in turn, results in different estrogenicities: 17α-estradiol is less estrogenic than E2.[64,234]

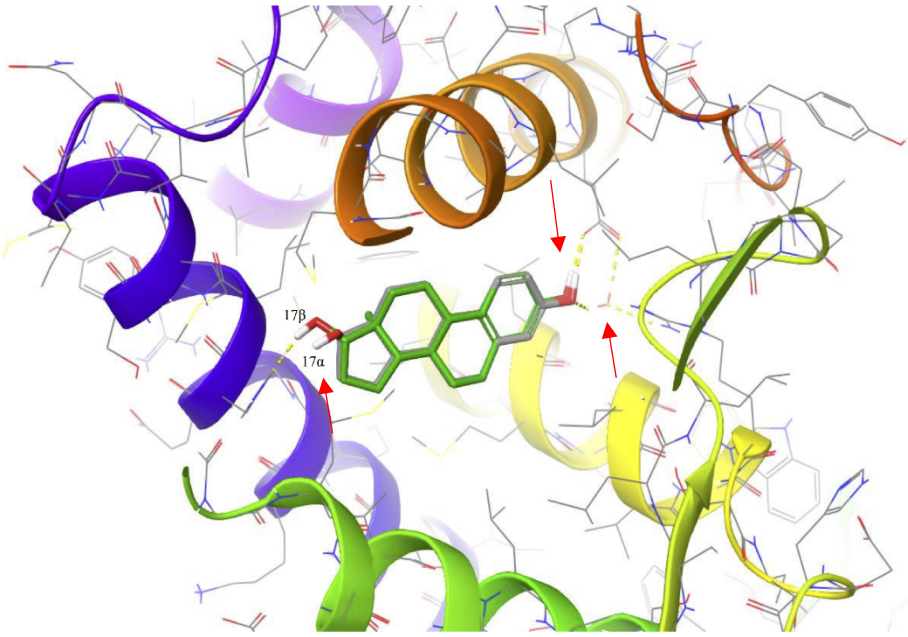
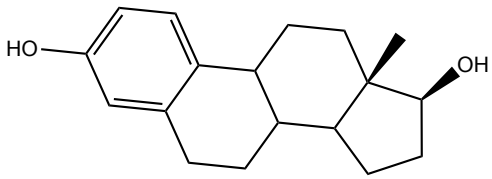
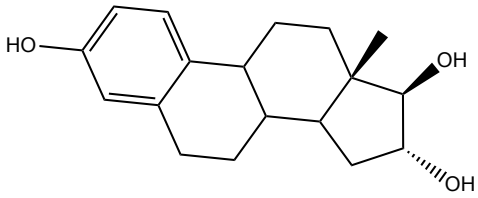
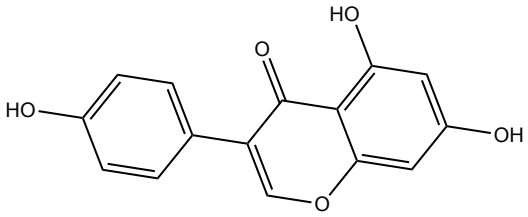
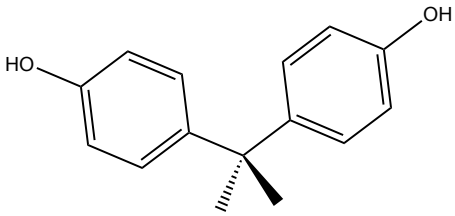


Figure 4.1: Overlay of 17α-estradiol and E2. E2 and 17α-estradiol bind at the LBC of ERα *via* H-bonds (---,Indicated by red arrows). Helices are represented by ribbons.

In addition, Chapter 3 introduced the potential structure-binding affinity/activity relationships of ligands with ERα; ligands with different structural features have different binding energies and binding affinities resulting in correspondingly different levels of estrogenicities. This is due to the presence and strength of the noncovalent interactions which are determined by the relative locations and spatial arrangements of a ligand with specific amino acid residues in the LBC.[20] This, in turn, influence ligands estrogenicities. For example, published data show that whiles E2 and E3 have great structural similarities yet a different arrangements of hydroxyls; this results in different binding affinities and estrogenicities, and suggests that the structures of ligands could determine their estrogenicities (Table 4.1).[235] In addition, GEN and BPA present different molecular skeletons which are likely to be the hydrophobic core of the ligand that interacts with the hydrophobic region of the LBC (***Section 1.2.2, Chapter 1***), thus resulting in different RBAs with ER α and relative agonistic activities (RAAs) (Table 4.1).[235]

Table 4.1: Examples of exogenous estrogenic compounds and xenoestrogens.[235]

Compound	Structure	RBA	RAA
E2		100	100
E3		9.41	17.6
GEN		1.55	0.064
BPA		0.66	0.018

4.1.2 Soy isoflavones

A recent World Health Organisation report assessed the state of the science of endocrine disrupting chemicals (EDCs) and reported that there are over 8000 known EDCs and potentially many more.[236] A large proportions of these EDCs are estrogen mimics; they can be natural components of food (e.g., GEN and naringenin), synthetic medicinal or industrial

product (e.g., EE2 and methylparaben).[237] Xenoestrogens are classified as synthetic estrogens (e.g., BPA in polycarbonate plastic) or phytoestrogens (e.g., GEN in soy beans).[17, 209] Soy isoflavones are the main dietary phytoestrogens, and have been used medicinally in hormone replacement therapy to alleviate menopause symptoms.[13, 124, 189] However, as mentioned before (***Section 1.2.3, Chapter 1***), the environmental exposure to these compounds may cause ERα-mediated adverse effects at a population level, such as reduced sperm count,[225] and might lead to precocious puberty in girls,[226] because female puberty requires an estrogen stimulus.[227] Interestingly, GEN and DAID can be biotransformed to hydroxylated metabolites, which have been widely detected in fermented soy foods (Fig. 1.12).[157] These metabolites have the same hydrophobic molecular skeletons as their parent compounds (estrogen mimics) but with different substituent arrangements (i.e. hydroxyls; Fig. 1.12), which might facilitate H-bond interactions with amino acid residues. This indicates the metabolites of isoflavones fit the binding requirements for interacting with ERα, but might facilitate different noncovalent interactions with the LBC resulting in different ERα-driven effects. This hypothesis will be studied in this chapter.

4.1.3 Bio-assays for estrogenicity

To determine the estrogenicity of environmental chemicals and dietary components, bio-assays have been used.[238] Receptor-gene assays use transgenic human cell lines or yeast cells;[239] that have been co-transfected with complementary DNA (cDNA) and a reporter gene containing an ERE.[240] In addition, in these type of assays, yeast or a mammalian cell line lacking endogenous ER is transfected with an expression plasmid carrying the ERα, ERβ cDNA or any desired receptor variant together with an ER-inducible promoter or the ERE linked to a chloramphenicol acetyl transferase (CAT) or luciferase reporter cDNA. Addition of ER ligands induces dose-dependent transcription of the reporter protein CAT or luciferase and can easily be monitored and quantified.[241, 242] Another type of induced proliferation test employing estrogen-responsive target cells (e.g., breast cancer cells) is a common method to assess the estrogenicity of compounds.[243] For example, the E-Screen uses breast cancer cells

(MCF-7) which are exposed to a range of concentration of the test compound and cell proliferation monitored. In each E-Screen test, a dilution series of E2 (0.1 pM–1000 pM) is set up as a positive control, and the cells treated only with phenol red (an estrogen mimic, Fig. 4.2) free medium (e.g., phenol red-free RPMI-1640 medium) as a negative control.[202] Following the incubation period, cells are treated with trichloroacetic acid to precipitate proteins which is then stained with sulforhodamine B. The absorbance (492 nm) of protein bound with dye is measured; this quantitatively corresponds to the cell number.[202] Either by counting cells or determining protein levels as a surrogate for cell number. However, the limitation is mitogens other than estrogens also can influence the cell proliferation thus rendering non-specific responses by estrogens.[244]

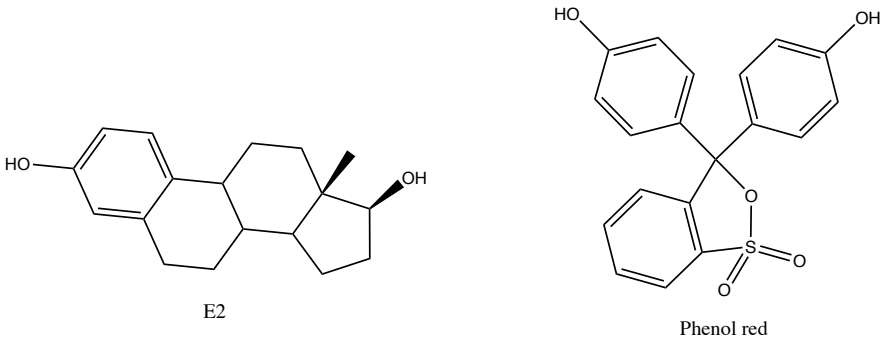


Figure 4.2: Molecular structure of E2 and phenol red

In addition, the binding affinity determines the strength of the interaction between a ligand and its receptor (e.g., estrogens and ERα), which, in turn, determines the receptor-driven effect of candidate ligands.[217] Measurement of the binding of a ligand (e.g., a xenoestrogen) to ERα or ERβ is usually done by competitive binding assays with radioactively or fluorescently labelled E2.[245, 246] Competitive binding assays determine RBAs compared to a positive control (E2), and therefore enable a ranking and prioritization of several compounds.[246] However, competitive binding assays do not measure physiological responses (e.g., cell proliferation).[246]

Perhaps the ultimate bioassay is one that involves ligand interaction in cells expressing ERs. A good example of such an assay is the MELN (MCF-7 cells are transfected with ERE-Glob-Luc-SVNeo plasmid) assay. The MELN assay is a sensitive recombinant cell receptor gene assay designed to determine the estrogenic potency of test chemicals.[55] The MELN assay uses MCF-7 cells which express endogenous ERα that are stably transfected with only an estrogen-regulated luciferase gene driven by an ERE in front of the βGlobin promoter (ERE-βGlob-Luc) which modulates luciferase expression (Fig. 4.3). This, in turn, activates the production of bioluminescence from the luciferase reaction following the addition of luciferin (Fig. 4.4).[55] These MELN cells enable the detection of compounds that bind to ERα or interfere with the induction of ERα-mediated gene expression (Fig. 4.3).[55]

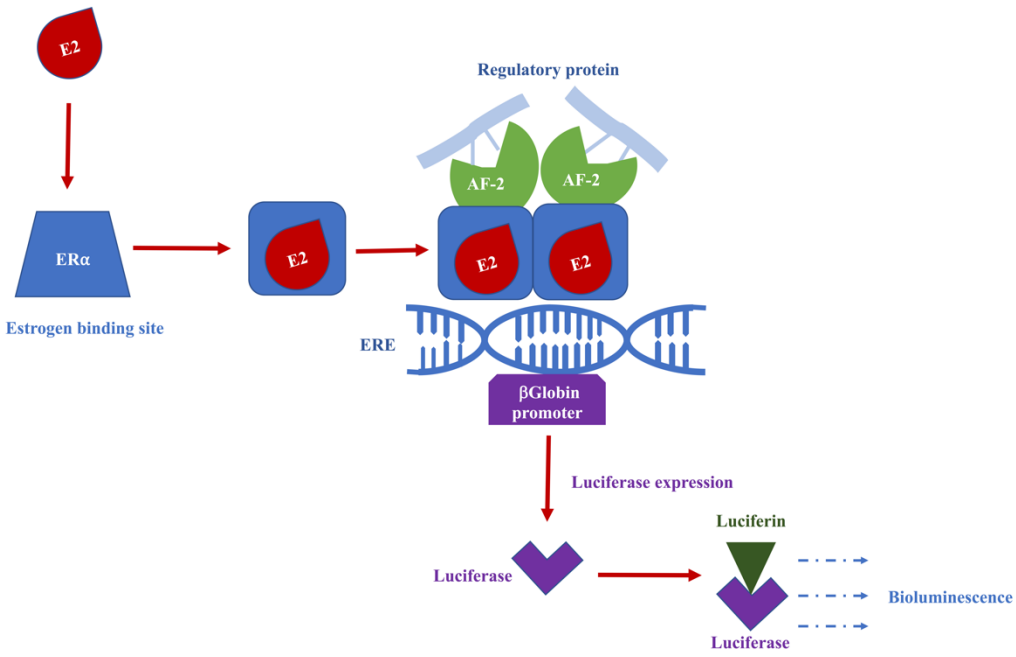


Figure 4.3: Schematic representation of the interaction between an estrogen (e.g., E2) and ERα resulting in induction of luciferase activity; this shows the working mechanism of MELAN assay. E2 binds to the LBC of ERα facilitating AF-2's hosting of a regulatory protein. After the dimerization of ERα, the dimer binds to ERE, which activates the β-Globin promoter resulting in expression of luciferase. Luciferase catalyses the luciferin reaction which produces bioluminescence. Bioluminescence is measured to quantify ERα occupancy.

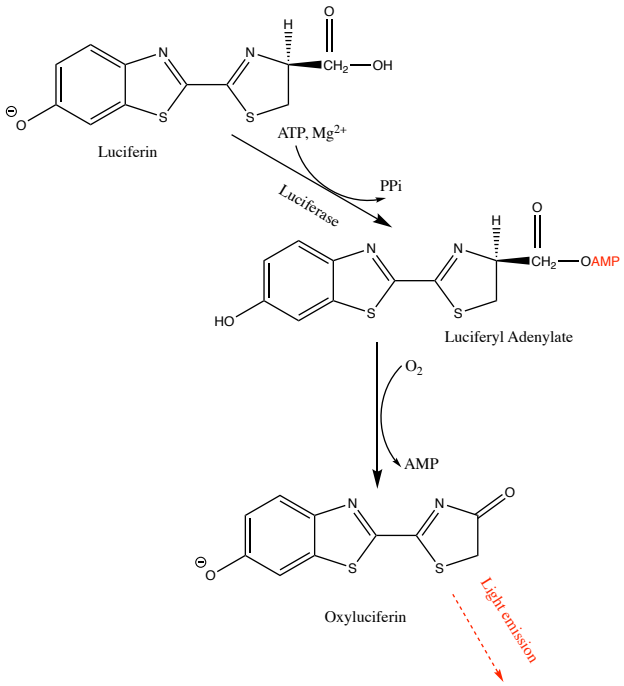


Figure 4.4: Bioluminescence reaction.

4.1.4 *In silico* study of the interaction between isoflavones and ERα

Chapter 3 introduced that *in silico* studies for understanding the possible receptor-mediated mechanisms that might be useful in drug discovery or exploring food component functionality in a receptor–ligand setting.[199] For example, *in silico* studies have been widely applied to the prediction of estrogenicity.[198, 247] Previous research reported a molecular docking system is used to model the interactions between anthraquinone derivatives (AQs, from plants, e.g., senna and rhabarb) and ERα (PDB ID:1X7R), and theoretical binding energy and estrogenicity have been successfully predicted.[198] Importantly, the predicted estrogenicity of AQs did not show a significant difference, compared with YES assay results.[198] In this chapter, the Schrödinger platform is used to investigate ERα interaction at a molecular level with selected ligands (i.e., isoflavones) and to study the theoretical binding energy of isoflavones with ERα. In addition, the MELN assay is used to measure the estrogenicity of the selected isoflavones.

4.2 Research objectives

The objectives of the research described in this chapter are:

- Use a computational bio−molecule platform (Schrödinger) to investigate the potential binding energy/affinity of selected isoflavones with ERα.

- Use the MELN assay to study the structure ERα-driven bioactivity relationship of selected isoflavones.

- Investigate the link between binding energy of ligands with ERα and estrogenicity.

4.3 Experiments

4.3.1 *In silico* modelling studies

The molecular docking studies were carried out as described before (***Section 3.3.1, Chapter 3***). The protein preparation and the receptor grid was prepared as described before (***Section 3.3.1, Chapter 3***). A single water molecule was also included in the protein-ligand complex as the triumvirate of the Leu-387, Arg-394 and H_2O interacts by H-bond with the 3′hydroxyl of GEN (Fig. 4.5). This water molecule is important for ligand–receptor interactions, but other water molecules were removed.[209]

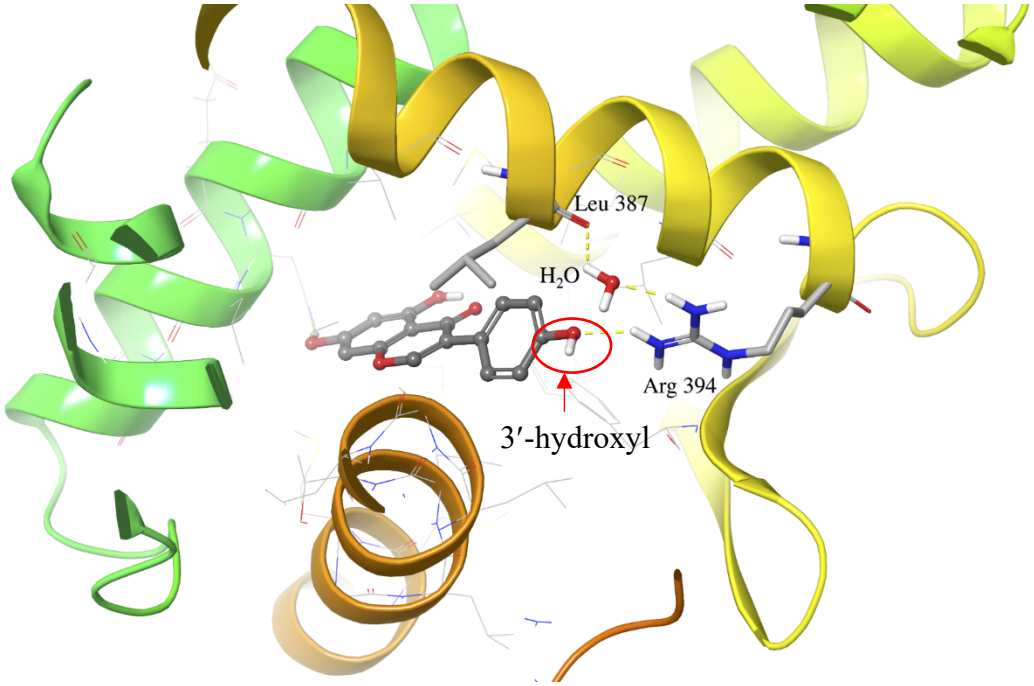


Figure 4.5: X-ray crystal structure of GEN in the binding pocket of ERα showing the triumvirate of the Leu-387, Arg-394 and H_2O interacting *via* a H-bond with the 3′-hydroxyl of GEN (shown in a red ellipse). H-bond interactions are represented by yellow dashed lines. Helices are represented by ribbons.

4.3.2 Preparation of ligands

Ligands (isoflavones shown in Table 4.2) were built and prepared using LigPrep (Schrödinger Release 2017-1: LigPrep, Schrödinger LLC, New York, NY, 2017).

4.3.3 Ligand docking and calculations.

Rigid receptor–flexible ligand docking calculations were performed using Glide in Extra Precision (XP) mode, and the process is same as descried in ***Section 3.3.4 Chapter 3***.

4.3.4 The MELN assay of isoflavones

These studies were carried out in collaboration with Katherine Trought, and carried out in her laboratory (Landcare Research, Lincoln).

4.3.4.1 MELN cell culture

All culture media, experimental reagents, and isoflavones stock solutions were prepared as described in ***Chapter 2***. MELN cells were obtained from INSERM, Montpellier France. They were routinely cultured in phenol red free RPMI-1640 medium containing 10% v/v stripped FBS, at 37 °C, 95% relative humidity and a CO_2 concentration of 5% v/v in air.

MELN cells were seeded into 96-well tissue culture plates (2.5×10^5 cells/mL) with 200 µL phenol red free RPMI-1640 medium containing 10% v/v stripped FBS. After 24 hours, the medium was replaced by fresh phenol red-free RPMI-1640 medium. A dilution series of the individual isoflavones was made in DMSO and 1 µL added per well (final concentrations are: 5×10^{-6} M, 1.25×10^{-6} M, 3.13×10^{-7} M, 7.81×10^{-8} M, 1.95×10^{-8} M, 4.88×10^{-9} M, 1.22×10^{-9} M, 3.05×10^{-10} M). E2 was used as the standard, the final concentrations are: 7.60×10^{-14} M, 3.05×10^{-13} M, 1.22×10^{-12} M, 4.88×10^{-12} M, 1.95×10^{-11} M, 7.81×10^{-11} M, 3.13

$\times 10^{-9}$ M, 1.25×10^{-9} M, 5.0×10^{-9} M in the well. Blanks were created by adding 1 µL DMSO per well. After 24 h incubation, the exposure medium was discarded, and replaced with 100 µL of media containing luciferin (25 mg/mL in DMSO). The plate was incubated for 5 min at 37°C and the luminescence was measured at 1 second intervals per well, with 7 readings per plate using a Varioskan Flash plate reader. Data were expressed in relative light units (RLU). The data was normalized by dividing the RLU response of experimental wells by the mean RLU of the corresponding blanks. Standards, positive controls (E2 at 1.9×10^{-11} M) and blanks were run on all plates. All the experimental compound responses were determined from three independent experiments.

The protein content of the MELN cells was measured by using a fluorescence assay, in which fluorescein was added to MELN cells in 96 wells cell culture plate, plated using the method described by Lorenzen and Kennedy,[248] to determine cytotoxic responses caused by the studied isoflavones. BSA was used to create a calibration graph (see Fig. S1, Appendix B). The detection limit was determined using three times the standard deviation (SD) of the solvent control, yielding detection limits of 1.1×10^{-12} M E2.

4.3.4.2 Data analyses

Raw data from MELN assays were analysed using Microsoft Excel® (Microsoft Corporation, Redmond, WA). Mean values ± SD were calculated. The average of the "no cell control" was subtracted from all mean values and supramaximal responses were removed. The data were evaluated by a nonlinear four-parameter logistic model to estimate the EC_{50} (concentration for 50% of maximal effect) using GraphPad Prism (Version 7). The RAA of each compound was calculated as the ratio of the EC_{50} value of each compound and the EC_{50} value of E2.

4.4 Results and Discussion

4.4.1 *In silico* study of the interactions between isoflavones and ERα

The *in silico* study predicts that all studied isoflavones can bind to the LBC of ERα. Table 4.3 shows that the individual isoflavones have the same molecular skeleton (Table 4.2) but with different hydroxyl substitution patterns, thus facilitating different noncovalent interactions with the LBC; this likely result in different H-bond values, HER, and thus different DockingScores which might influence the binding affinity of the ligands (Table 4.2). This indicates the potential contribution of structural features (e.g., hydroxyls arrangements) on the binding energy and binding affinity of isoflavones to ERα.

Table 4.2. Substitution positions, HB value, HER, and DockingScore of the studied isoflavones (in order of DockingScore).

Compound	Substitution position	H-bond value (kcal/mol)	HER (kcal/mol)	DockingScore (kcal/mol)
6-OH-DAID	4′, 6, 7 (-OH)	−2.42	−4.99	−11.99
3′-OH-GEN	3′, 4′, 5, 7 (-OH)	−2.59	−4.71	−11.8
8-OH-GEN	4′, 5, 7, 8 (-OH)	−2.5	−4.6	−11.68
8-OH-DAID	4′, 7, 8 (-OH)	−2.27	−4.97	−11.62
3′-OH-DAID	3′, 4′, 7 (-OH)	−2.13	−5.01	−11.5
GEN	4′, 5, 7 (-OH)	−1.7	−4.8	−10.79
DAID	4′, 7 (-OH)	−1.26	−4.90	−10.49
FOR	4′ (-OH); 7 (-OCH_3)	−0.64	−5.23	−9.78

As described before (***Section 3.4.4, Chapter 3***), H-bond value is the sum of the individual H-bond energies between a ligand and the receptor which is determined by both distribution and orientation of the ligand's hydroxyls that might form H-bonds with amino acid residues in the LBC under a ligand–receptor situation.[217] Table 4.2 shows that the studied isoflavones have different arrangements (i.e., number and positions) of hydroxyls on their molecular skeletons, and have different H-bond values. This suggests the H-bond value could be affected by the number of hydroxyls on the ligand; for example, the H-bond value of DAID (dihydroxyisoflavone; −1.26 kcal/mol) < GEN (trihydroxyisoflavone; −1.7 kcal/mol) (Table 4.2). However, some isoflavones with the same number of hydroxyls have different H-bond values; for example, both 6-OH-DAID and 3′-OH-DAID are tri-hydroxy isoflavones, but the

H-bond value of 6-OH-DAID is −2.42 kcal/mol and 3′-OH-DAID is −2.59 kcal/mol. This could be due to their different hydroxyl orientations resulting in different H-bond interactions with ERα (Fig. 4.6).

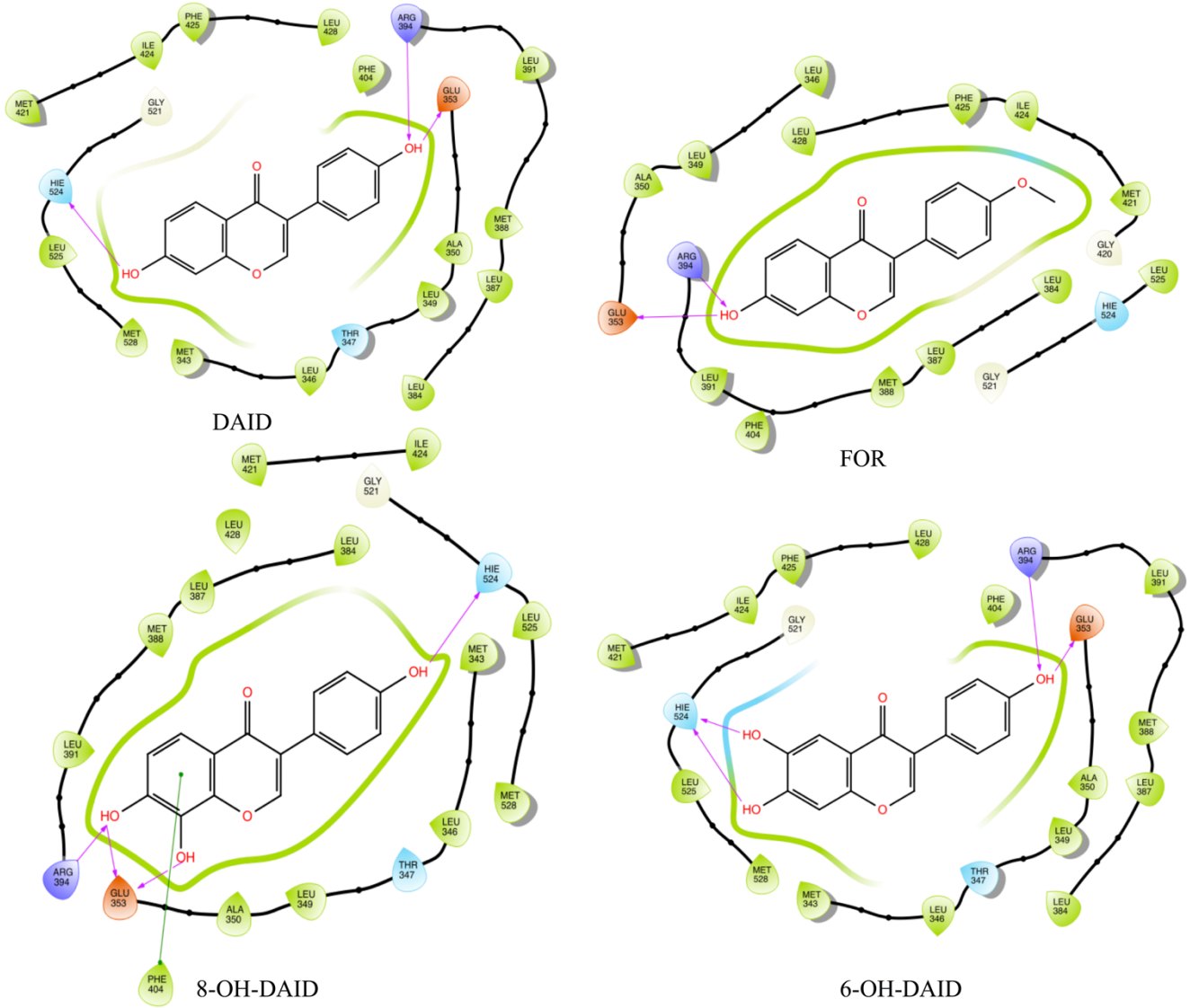


Figure 4.6: See the next page for legend.

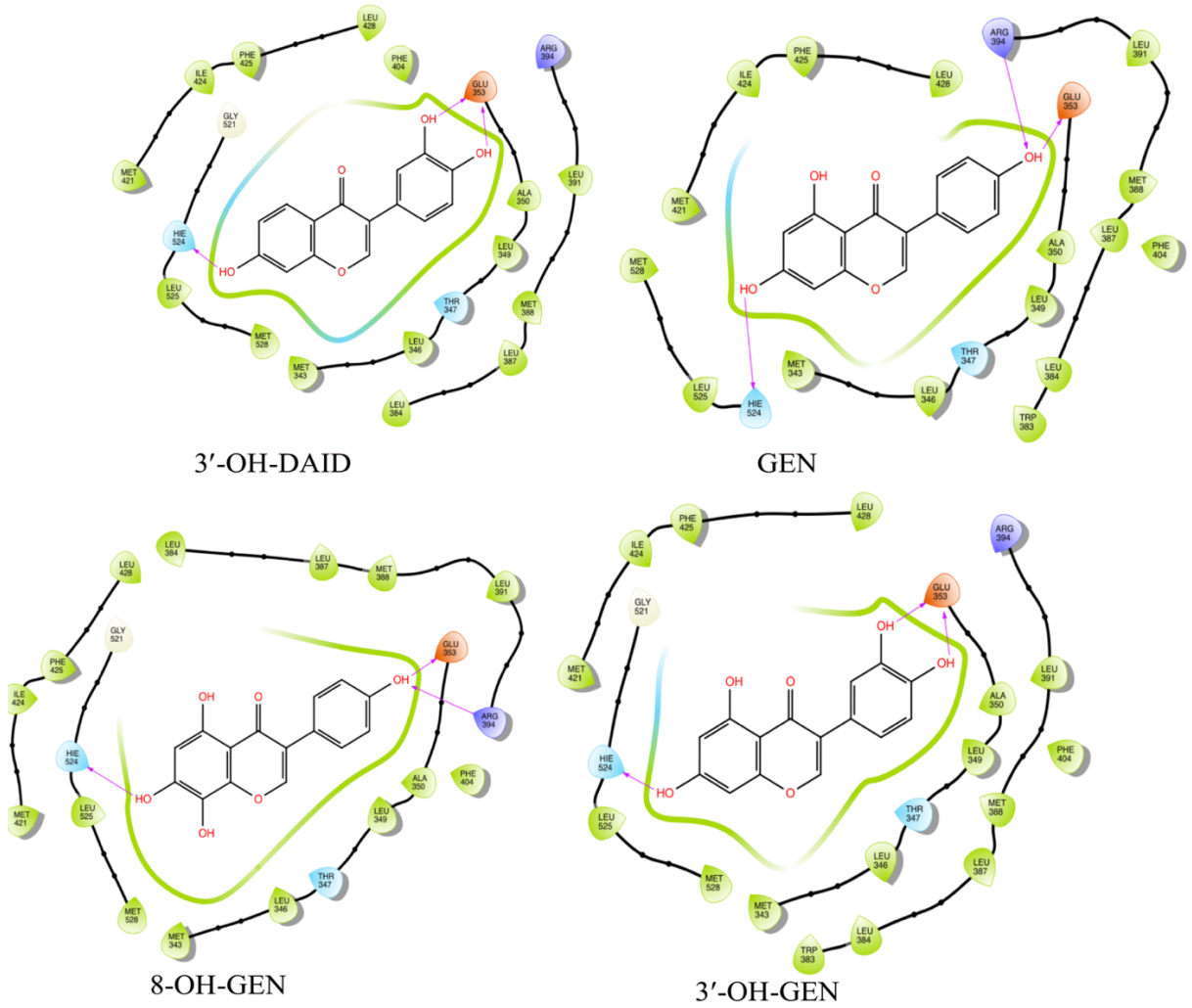
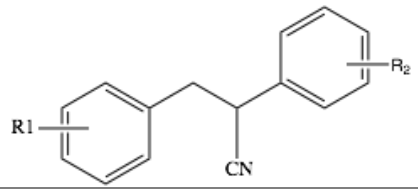


Figure 4.6: The H-bond interactions between ligands and amino acid residues in the LBC of ERα visualised in Schrödinger. Purple arrows represent H-bonds; the direction of arrow denotes electron donor to electron acceptor in the H-bond. Different coloured residues represent amino acid properties: blue = positive charge; red = negative charge; cyan = polar; green = nonpolar. (NB: Schrödinger uses a non-standard amino acid abbreviation system: HIE = Histidine).

In addition, the methoxy group of FOR leads to steric hindrance that also affects the ligand binding energy to ERα. This hindrance was predicted to force the methoxy hydroxyl groups to interact with different amino acid residues, which is shown in Figure 4.6, thus resulting in a lower DockingScore for FOR (−9.78 kcal/mol) compared with DAID (−10.49 kcal/mol), and thus a lower binding affinity. Interestingly, published results of a competitive radiometric binding assay with [^{3}H]-E2 shows 2,3-bis (4-hydroxyphenyl) propionitrile (DPN) has a higher RBA compared with its methylated derivate (Table 4.3);[173] this is likely due to the stearic hindrance caused by the methylated hydroxyl group, forcing the methylated derivate to interact with ERs at a unfavourable orientation, resulting in lower RBA with ERs.

Table 4.3. RBA of DPN and its methylated derivate with ERα and ERβ



Ligand	R1	R2	ERα	ERβ
DPN	*p*-OH	*p*-OH	0.25	18
Methoxy-DPN	*p*-H, *o*-Me	*p*-OH	0.87	60

As discussed before in ***Chapter 3***, HER is the term used to differentiate the relationship between the hydrophobicity of ligands and the 3 dimensional receptor environment.[208] This describes the spatial arrangement of hydrophobic amino acid residues (e.g., Leu) in a LBC of; for example, a receptor (in this case ERα) that supports hydrophobic interactions between a ligand and the receptor.[208, 206] Table 4.2 shows that the isoflavones studied have similar HER (range from −4.6 to −5.23 kcal/mol); this could be due to the same hydrophobic region (Fig. 4.6). However, the different arrangements of hydroxyls appear to affect the HER. For

example, the HER of DAID (−4.9 kcal/mol) is less than the corresponding values for FOR (−5.23 kcal/mol), because the hydroxyl is more polar than the methoxyl, resulting in less hydrophobicity of DAID. In addition, the HER of 8-OH-GEN (−4.6 kcal/mol) is less than that for 3′-OH-GEN (−4.71 kcal/mol) as 8-OH-GEN has more free hydroxyls, which cannot form H-bonds with amino acid residues in the LBC. These σ^- free hydroxyls are not favourable in the hydrophobic binding region of the LBC and resulting in a reduction in 8- OH-GEN's hydrophobicity (Fig. 4.6). Likewise, Table 4.1 shows E3 has an extra hydroxyl compared with E2 and resulting in a lower RBA (9.41) and correspondingly lower RAA (17.6);[235] this also suggests the extra hydroxyl could cause unfavourable interaction with the LBC of ERα, and, thus decrease the RBA of the ligand with ERs. This might result in a corresponding decrease in estrogenicity.[235] The docking study of isoflavones described here provides important information about how non-steroidal estrogenic compounds are oriented in the LBC, and shows that the potential effects of polar groups on ligand binding depend on both the relative position on the skeletal ring structure and their spatial orientations thus facilitating with the specific interactions with amino acid residues in the LBC.

4.4.2 Estrogenicity of isoflavones

The results of fluorescence assay show there is not cytotoxic responses caused by the studied isoflavones on MELN cells. The estrogenicities of the selected eight isoflavones were determined using the MELN assay. Figure 4.7 shows that the isoflavones cause different estrogenic responses in the MELN assay and that the responses are dose-dependent. The EC_{50} and RAA of the selected isoflavones are shown in Table 4.4, all the isoflavones studied are weakly estrogenic (the RRAs of isoflavones are at least 10^4 times less than E2, where E2 = 1). Hydroxylated metabolites of GEN and DAID showed greater RAA in the MELN assay compared with their less hydroxyl parent compounds (Table 4.4). For example, the RAA of GEN is 1.1×10^{-5}, which is less than 3′-OH-GEN, its RAA is 1.7×10^{-5}; the RAA of DAID is 1.5×10^{-6}, which is less than 3′-OH-DAID, its RAA is 2.3×10^{-5} (Table 4.4).

However, the exception to this trend is 8-OH-GEN, which has a significant lower RAA than GEN. This might be due to 8-OH-GEN's additional hydroxyl groups of (Fig. 4.6) which means that there are no free potential H-bonding amino acid residues in the vicinity of the docked ligand, the additional hydroxyl cannot form H-bonds with the LBC (Fig. 4.6). In addition, the extra hydroxyl groups would have unfavorable interactions with the hydrophobic region of the binding cleft.

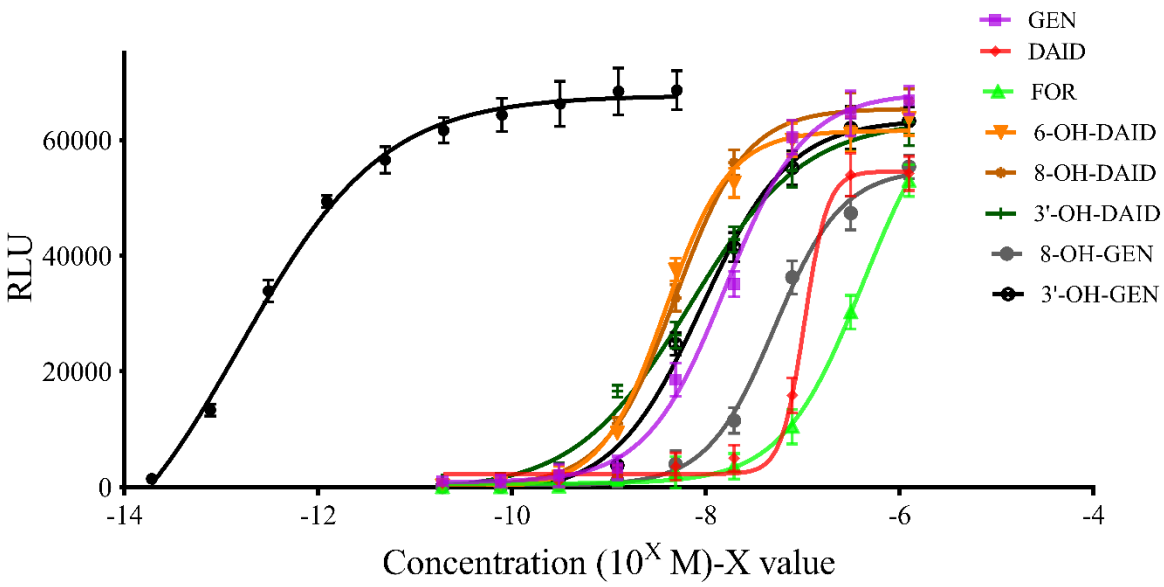


Figure 4.7: MELN assay of isoflavones. Mean ± SD are shown.

Table 4.4. Comparison of EC_{50} and RAA (determined by MELN assay) of isoflavones.

Compound	EC_{50} (M)	RAA
E2	1.5×10^{-13}	1.0
6-OH-DAID	3.8×10^{-9}	4.0×10^{-5}
8-OH-DAID	4.8×10^{-9}	3.2×10^{-5}
3′-OH-DAID	6.8×10^{-9}	2.3×10^{-5}
3′-OH-GEN	9.1×10^{-9}	1.7×10^{-5}
GEN	1.4×10^{-8}	1.1×10^{-5}
DAID	1.0×10^{-7}	1.5×10^{-6}
8-OH-GEN	5.1×10^{-8}	3.0×10^{-6}
FOR	4.2×10^{-7}	3.6×10^{-7}

4.4.3 Structure activity relationship of isoflavones

Structure activity relationship links structural features of molecules to their biological activities.[249] The interaction between ligands and ERα, and the corresponding receptor-driven activity is a good example;[72] this ligand–receptor system requires specific binding characteristics (e.g., binding affinity and binding energy), which are determined by the

relative spatial arrangement of a ligand's functional groups (e.g., hydroxyl, keto) and the docked ligand would be predictable in an *in silico* molecular modelling system (e.g., Schrödinger).[250] *In silico* study is a crucial facet of drug design, used by the pharmaceuticals industry today.[250] Even without sophisticated molecular modelling software, it is possible to predict pharmacological properties related to key functional groups on a molecule by comparing activities of closely related structures (i.e., structure–activity relationships).[218]

In this study, the estrogenicity of eight isoflavones was studied. Most of the hydroxyl-metabolites have higher DockingScores than their corresponding parent compounds; this predicts the increase in estrogenic potency seen in the MELN assay (Table 4.4). The additional hydroxyls are likely to mean that form more H-bonds are found with specific adjacent amino acid residue in the LBC of ERα (e.g., His 524, Fig. 4.6). In turn, this may increase the binding energy and binding affinity of the hydroxylated metabolites to ERα. Indeed, the positive association between amount of hydroxyl groups of ligands and receptor-driven activity (i.e., estrogenicity) is supported by published work that show that based on the same ligand-receptor system; previous research showed biochanin A (having an extra hydroxyl than FOR, Fig. 4.8) is more estrogenic than FOR.[223]

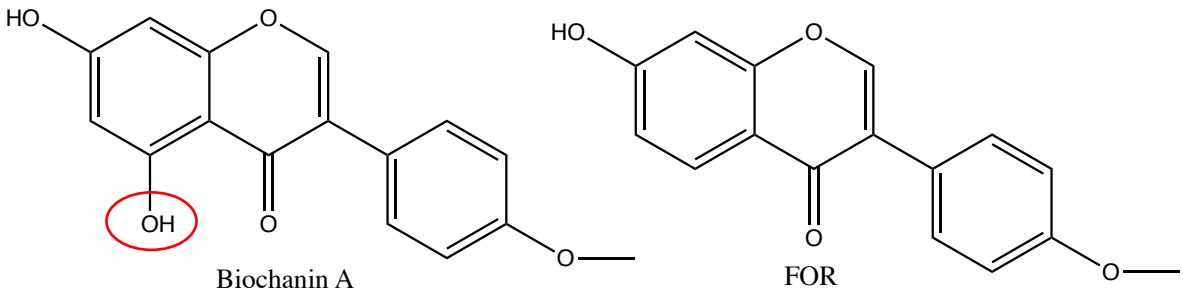


Figure 4.8: Structural comparison between biochanin A and FOR showing biochanin A has an extra hydroxyl group (circled in red) which might explain why it is more estrogenic than FOR.

Intriguingly, 8-OH-GEN does not fit the positive correlation trend between DockingScore and estrogenicity discussed above. The Schrödinger modelling system predicts that 8-OH-GEN would have a higher binding energy than GEN; the DockingScore of 8-OH-GEN (−11.68 kcal/mol) is higher than GEN (−10.79 kcal/mol). This implies 8-OH-GEN would be more estrogenic than GEN. However, the MELN assay shows 8-OH-GEN (RRA: 3.01×10^{-6}) has weaker estrogenicity than GEN (RRA: 1.70×10^{-5}). This is likely due to the two free hydroxyls on 8-OH-GEN (Table 4.2) which are unable to form H-bonds with amino acid residues in the LBC (Fig. 4.6) and reducing the hydrophobicity of 8-OH-GEN, and thus influencing its estrogenicity. Previous research shows, this is similar to the weaker estrogenicity of morin ($EC_{50} = 2.4 \times 10^{-7}$ M) compared to apigenin ($EC_{50} = 2.4 \times 10^{-6}$ M).[190] Again, the extra hydroxyl of morin might be not favourable in the hydrophobic region of the LBC of ERα, thus resulting in lower estrogenicity compared to the less hydroxylated compound apigenin (Fig. 4.9).

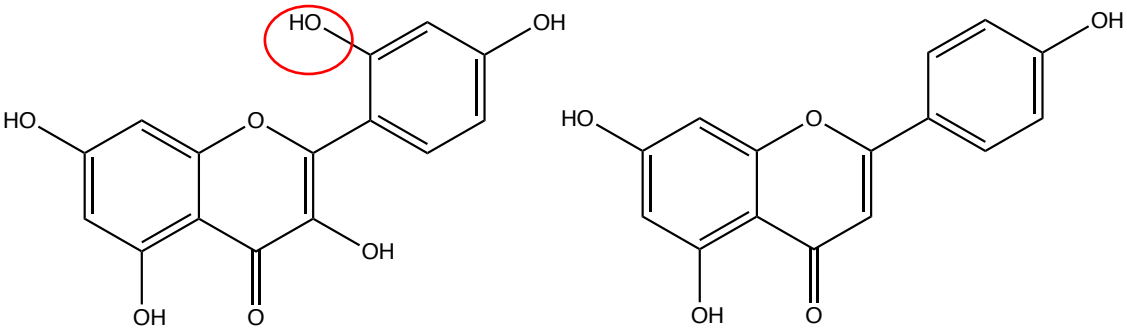


Figure 4.9: Structural comparison between morin and apigenin showing morin has two additional hydroxyl groups (circled in red) which might explain its more estrogenic compared with apigenin.

4.5 Conclusions

In this chapter, the Schrödinger platform is used to predict the binding energy and order of binding affinity of GEN, DAID and their hydroxylated metabolites with ERα. This *in silico* study indicates that the number and orientation of hydroxyls on ligands could affect the binding energies and resulting in different binding affinities. Results of the *in silico* study presents a trend: more hydroxyls might form more H-bonds resulting in a higher binding energy with the receptor. However, the free hydroxyl groups of ligands, which cannot form any polar interactions (e.g., H-bonds) with amino acid residues in the hydrophobic region of the LBC; this might decrease the hydrophobicity of the ligand resulting in unfavourable contact with the LBC of ERα, thus reducing binding energy and influencing estrogenicity. These results are backed up by the MELN assay: hydroxylated metabolites of GEN and DAID exhibit greater estrogenicity than their corresponding precursors. However, 8-OH-GEN is an exception which does not follow this trend; this compound is predicted to have a greater binding affinity than the parent compound GEN, but surprisedly showed a weaker RRA in the MELN assay compared to GEN. This likely results from the unfavorable interactions of the free hydroxyls of 8-OH-GEN with the hydrophobic region of the LBC. Hydroxylated metabolites of DAID and GEN are produced from food fermentation and have different estrogenicities compared with their parent compounds. The type of metabolite depends on the specific microorganism used in the fermentation;[157, 251, 252] which is due to specific CYP450 expressed by the microorganism.[253, 254] In short, this suggests a potential link between food science technology (e.g., food fermentation) and functional components (e.g., isoflavones and their fermentation metabolites) of foods. The raw materials used in the fermentation process can be converted into different metabolites (e.g., hydroxylated isoflavones); these metabolites have different levels bio-activities (e.g., estrogenicities), and this determines food functionality.

Chapter 5: The ERβ-mediated effects of isoflavones on the proliferation of Caco-2 cells

5 Chapter 5 – The ERβ-mediated effects of isoflavones on the proliferation of Caco-2 cells

5.1 Introduction

5.1.1 Distributions and functionalities of ERs

Steroid hormones are important in control mechanisms for many different biological functions depending on the distributions of individual isoforms of steroid hormone receptor, including, but not limited to, metabolism, inflammation, proliferation and development, immune function, and reproduction.[51, 255, 173] As previously discussed in ***Chapter 1***, ERα is mainly found in the central nervous system, liver, breast and ovary; and plays an important role in maintaining the cell proliferation in these organs or tissues.[12, 52] For example, the regulation of ERα in the central nervous system has been linked with energy homeostasis, which is associated with obesity.[256] Obesity has been increasing sharply in industrialized countries since the last century and is now recognized as a global epidemic; Over one-third of Americans are obese, and the population of obesity in developing countries (e.g., China and Thailand) is increasing quickly.[257] A decrease in estrogen signalling caused by ageing or gonadectomies increases body weight and central (abdominal cavity) adiposity,[256] and mice with the ERα gene knockout from the brain (by gene-targeting techniques) showed central adiposity.[258]

ERβ has little importance in reproductive processes, particularly the proliferation of the cell of mammary glands or endometrium, and ERβ is not expressed in the pituitary which controls the development and growth of germ cells.[52] However, ERβ has been found in the cardiovascular system, urogenital tract, and GI tract.[52, 259] Interestingly, the incidence of cardiovascular disease differs significantly between men and women,[260] this suggests that, in part, estrogen levels might influence the risk factor of cardiovascular disease. In addition, the

incidence of atherosclerotic diseases is low in premenopausal women (higher average estrogen level), rises in postmenopausal women (lower estrogen level), and is reduced to premenopausal levels in postmenopausal women who receive estrogen therapy.[261] This could be explained by a previous research, which shows estrogen increases vasodilatation and inhibits the response of blood vessels to injury and the development of atherosclerosis.[261] Wild-type ovariectomized female mice and mice lacking ERβ were treated with E2, which showed that a lowering of hypertrophy in females is associated with ERβ.[259, 262] In addition, the fact that ERβ is the predominant ER isoform in the GI tract (e.g., epithelium, vascular smooth muscle, and endothelium) draws more attention to the potential interaction between ERβ and GI physiology.[64, 259, 263] Evidences of the impact of female hormones on GI physiology from observation of several functional GI disorders suggested considerable gender-specific epidemiological differences.[259, 264] For example, the female/male ratio for slow-transit constipation is approximately 9:1; which suggests this might be linked to the higher prevalence of irritable bowel syndrome in women than in men because of the important role of the estrogen regulation in the GI tract.[265] This 'gender bias' suggests the involvement of female hormones in the regulation of GI motility, which has been supported by the delayed GI transit time during pregnancy (high E2 and progesterone) compared with women during the menstrual cycle (corresponding low E2 and progesterone, except the follicular phase).[266]

Importantly, ERβ-mediated effects in the colon can extend to pathological conditions; for example, results of the rodent models of chronic intestinal inflammation show that an increase in colonic permeability is associated with a decrease in ERβ mRNA,[259, 267, 268] this may lead directly to gut diseases (e.g., colon cancer, inflammatory bowel disease).[64, 263]

5.1.2 The potential selective-tissue bioactivities of isoflavones *via* binding ERs

Section 1.2.5 Chapter 1 introduced the overall homology of the LBD between ERα and ERβ is surprisedly less than 55%; however, the LBC, which interacts directly with ligands, these

two isoforms of ER are remarkable homologous. This indicates that the two isoforms of ER might provide similar binding environment for ligands (Figs. 1.7 & 1.8).[54 173] Intriguingly, previous research shows that the binding affinities of ligands with the two isoforms of ER are different, and soy isoflavones (a main source of phytoestrogens) have a higher binding affinity to ERβ compared with ERα.[64] For example, the RBA of GEN and DAID interacting with ERα are 4 and 0.1, but with ERβ are 87, and 0.5 respectively, where E2's binding affinity is 100.[64] This suggests the relative ERβ-mediated bioactivity of GEN and DAID in ERβ-predominated organs (e.g., gut cells) would be greater than the relative bioactivity in ERα-predominated organs (e.g., human breast); where E2 is set up as a standard compound.[12]

The Caco-2 cell line is a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma cells from the GI tract.[269] Published research shows that the proliferation of Caco-2 cell cultures were stimulated with either E2 or GEN alone, but was inhibited by either E2 or GEN in the presence of ICI 182,780 (Fig. 5.1), which is a pure antagonist of ERs.[270] In addition, ERβ is the predominated isoform of ER in Caco-2 cells.[52, 270, 271] Bring the statements in this section together, the proliferative effects of E2 and GEN on Caco-2 cell line is ERβ mediated.[52, 270, 271]

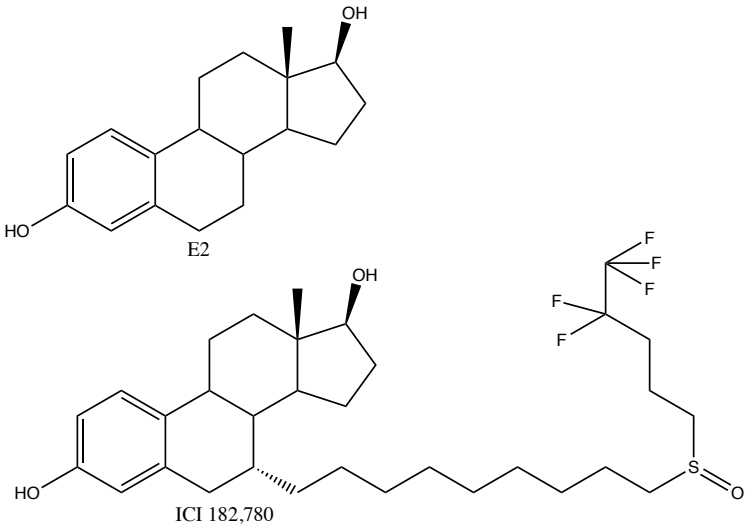


Figure 5.1: The structural comparison between E2 and ICI 182, 780.

The hydroxylation in food fermentation processes converts two estrogen mimics—GEN and DAID into their hydroxylated metabolites, this potentially increases the number of H-bonds of ligands with the LBC and resulting in greater binding affinity (***Section 1.5.2, Chapter 1***).. In addition, the MELN assay in ***Chapter 4*** shows these hydroxylated metabolites have different ERα-driven activity.

5.2 Research Objectives

The objectives of the research described in this chapter are:

- Use a computational bio−molecule platform (Schrödinger) to study the theoretical binding energy and binding affinity of selected isoflavones (including GEN, DAID and their hydroxylated metabolites) with ERβ.

- Study the effects of selected isoflavones on the proliferation of Caco-2 cells, and investigate their potential structure-activity relationships.

- Investigate the potential influence of the intestinal phase II metabolism on the ERβ-driven bioactivity of selected isoflavones.

5.3 Experiments

5.3.1 *In silico* modelling studies

The molecular docking studies were carried out as described before (***Section 3.3.1, Chapter 3***). The protein preparation and the receptor grid was adapted from ***Section 3.3.1, Chapter 3***). The X-ray crystallographic coordinate of ERβ was taken from the PDB , and the X-ray crystal structure of ERβ complexed with GEN (PDB entry 1X7J) was used (Fig. 5.1).[209] This crystallize structure of ERβ has two identical chains (Chain A and Chain B), with each chain having a docked ligand (i.e., GEN). Chain A was arbitrarily used as the ligand–receptor subunit for this docking study, and chain B was deleted. Missing amino acid residues (Ala 262, Tyr 411, Pro 412, Leu 413, Val 414, Thr 415, Ala 416, Thr 417, Gln 418, Asp 419, Ala 420, His 498, Val 499, Leu 500) were added to complete the protein structure using the Schrödinger 'Prime' command. Ligands (isoflavones) were built and prepared as described before (Section 3.3.3, Chapter 3). Rigid-receptor-flexible-ligand docking calculations were performed using XP mode and the process was same as the ***Section 3.3.4 Chapter 3***.

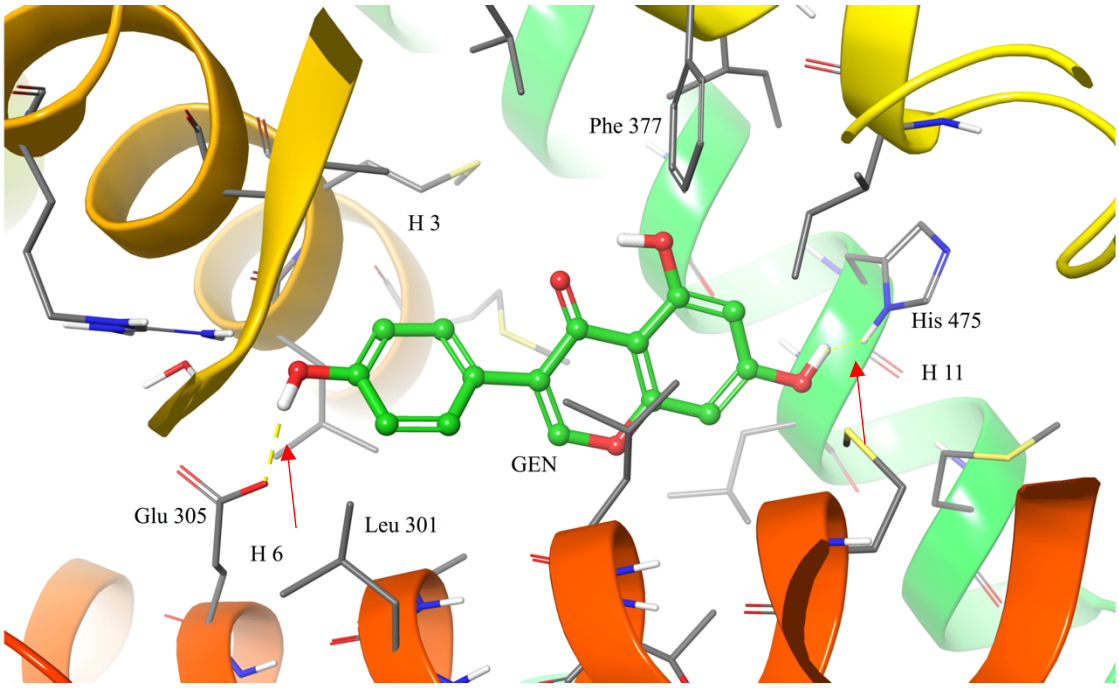


Figure 5.2: X-ray crystal structure of GEN in the binding pocket of ERβ (PBD entry code: 1X7J) visualised in Schrödinger showing the H-bonds (---, pointed out with a red arrow) between the ligand and LBC. Amino acid residues interacting with the ligand via H-bonds are shown. All amino acids residues within 3 Å of the ligand are also shown to indicate the chemical nature of the binding pocket.

5.3.2 Caco-2 cell proliferation studies

The Caco-2 (ATCC HTB-37) cell were a gift from Associate Professor Jacqui Keenan (Christchurch School of Medicine, University of Otago). The preparation of culture media, experimental reagents, and stock solutions of isoflavones are described in ***Chapter 2***.

5.3.2.1 Caco-2 cell culture

The cell culture method was adapted from Budd *et al.*[272] Caco-2 cells were cultured at 37ºC in a humidified atmosphere of 5% CO_2 v/v in air atmosphere in MEM with Earle's Salt and

L-glutamine, supplemented with 10% (v/v) heat-inactivated FBS, 0.1 mM MEM non-essential amino acids and 1% (w/w) penicillin/streptomycin.[272]

5.3.2.2 Isoflavone exposure experiments

The exposure concentrations (range from 5×10^{-8} M to 6.4×10^{-5} M) of isoflavones were prepared from the stock solutions in DMSO.[270, 273] The blank was set up with the same volume of chemical solvent in the absence of isoflavones and a positive control group consisted of E2 at concentrations ranging from 1.92×10^{-12} to 1×10^{-8} M. ICI 182,780 (1 µM) (Fig. 5.2),[274] an antagonist compound for ERs, was used as a negative control.[271] Briefly, the cell cultures were incubated with individual isoflavones. After 3 d, the culture medium was aspirated, and the cells were washed with 5 mL PBS followed by addition of 3 mL 2.5 % trypsin (w/v), and the culture plate was incubated for 15 min at 37ºC. Subsequently, 12 mL MEM containing 3 mL trypsin was added to the cell cultures and was thoroughly mixed to suspend the cells then the mixture was transferred to a 50 mL sterile centrifuge tube. The cell counting process is described as ***Section 2.5***.

5.3.2.3 GA treatment

Caco-2 cells were pre-treated with GA at 37.5 µM (GA's IC_{50}–half maximal inhibitory concentration value of Caco-2 cell is 68.7 µM) for 24 h, then cultured with selected isoflavones (GEN and 8-OH-GEN) at 0.5 µM for another 72 h.[275] Afterwards, the cells were treated with trypsin and counted as described above (***Section 5.3.2.2***).

5.4 Results and Discussion

5.4.1 *In silico* study of the interactions between isoflavones and ERβ

In the *in silico* study, seven isoflavones were docked at the LBC of ERβ. Table 5.1 shows that these isoflavones have different H-bond values, HERs, and thus different DockingScores. GEN and DAID, which are estrogen mimics,[210] were predicted to have lower binding energy and lower binding affinity than their hydroxylated metabolites (Table 5.1); this suggests these that the hydroxylated metabolites might interact with LBC and be estrogenic. In addition, the different H-bond values and HERs indicate the potential contribution of structural features (i.e., the distributions of hydroxyls) to the interaction of isoflavones with the LBC thus resulting in different DockingScores and binding affinities with ERβ (Tables 5.1 & 5.2).

Table 5.1: Substitution positions, H-bond value, HER, DockingScore of the studied isoflavones (in order of DockingScore).

Compound	Substations (-OH)	DockingScore kcal/mol	H-bond value kcal/mol	HER kcal/mol
DAID	7, 4'	−11.03	−1.21	−5.68
GEN	5, 7, 4'	−11.45	−1.67	−5.52
3'-OH-DAID	7, 3', 4'	−11.56	−1.72	−5.64
8-OH-GEN	5, 7, 8, 4'	−11.64	−1.66	−5.38
3'-OH-GEN	5, 7, 3', 4'	−11.72	−2.18	−5.47
8-OH-DAID	7, 8, 4'	−11.85	−1.92	−5.64
6-OH-DAID	6, 7, 4'	−11.88	−2.18	−5.57

As discussed in ***Section 3.4.4 Chapter 3*** H-bond value is determined by the orientation of individual H-bonds, which is based on both the angles and distance between the donor and acceptor atoms in the bond.[217] This means that H-bond values could be affected by both distribution and orientation of the ligand's (e.g., selected isoflavones) hydroxyls which might form H-bonds with amino acid residues in the LBC under a ligand/receptor docking situation. Table 5.1 shows these isoflavones have various arrangements of hydroxyls and different H-bond values; this indicates that an increasing trend in the H-bond values could be due to additional hydroxyls. For example, the H-bond value of DAID (−1.21 kcal/mol) < the H-

bond value of GEN (−1.67 kcal/mol) < the H-bond value of 3′-OH-GEN (−2.18 kcal/mol) (Table 5.1). Interestingly, some isoflavones have the same number of hydroxyls but show different H-bond values; for example, 6-OH-DAID (−1.72 kcal/mol) and 3′-OH-DAID (−2.18 kcal/mol). This likely result from the different hydroxyl arrangements between 6-OH-DAID and 3′-OH-DAID and the formation of different H-bond interactions with ERβ (Fig. 5.3). In addition, Figure 5. 3 shows a specific hydroxyl arrangement (i.e., 8-OH-DAID, Table 5.1) changes the ligand's relative orientation with ERβ, thus leading the hydroxyls (i.e., the hydroxyl at positions 7 and 8; Table 5.1) to interact with different amino acid residues in the LBC compared with DAID. An understanding of the importance of H-bond interactions in a ligand–receptor binary system might be supported by the higher RBA of triphenol pyrazole (Table 3.4) with ERα compared with monohydroxy- and dihydroxy-phenols,[197] which was discussed in ***Section 3.4.4, Chapter 3***.

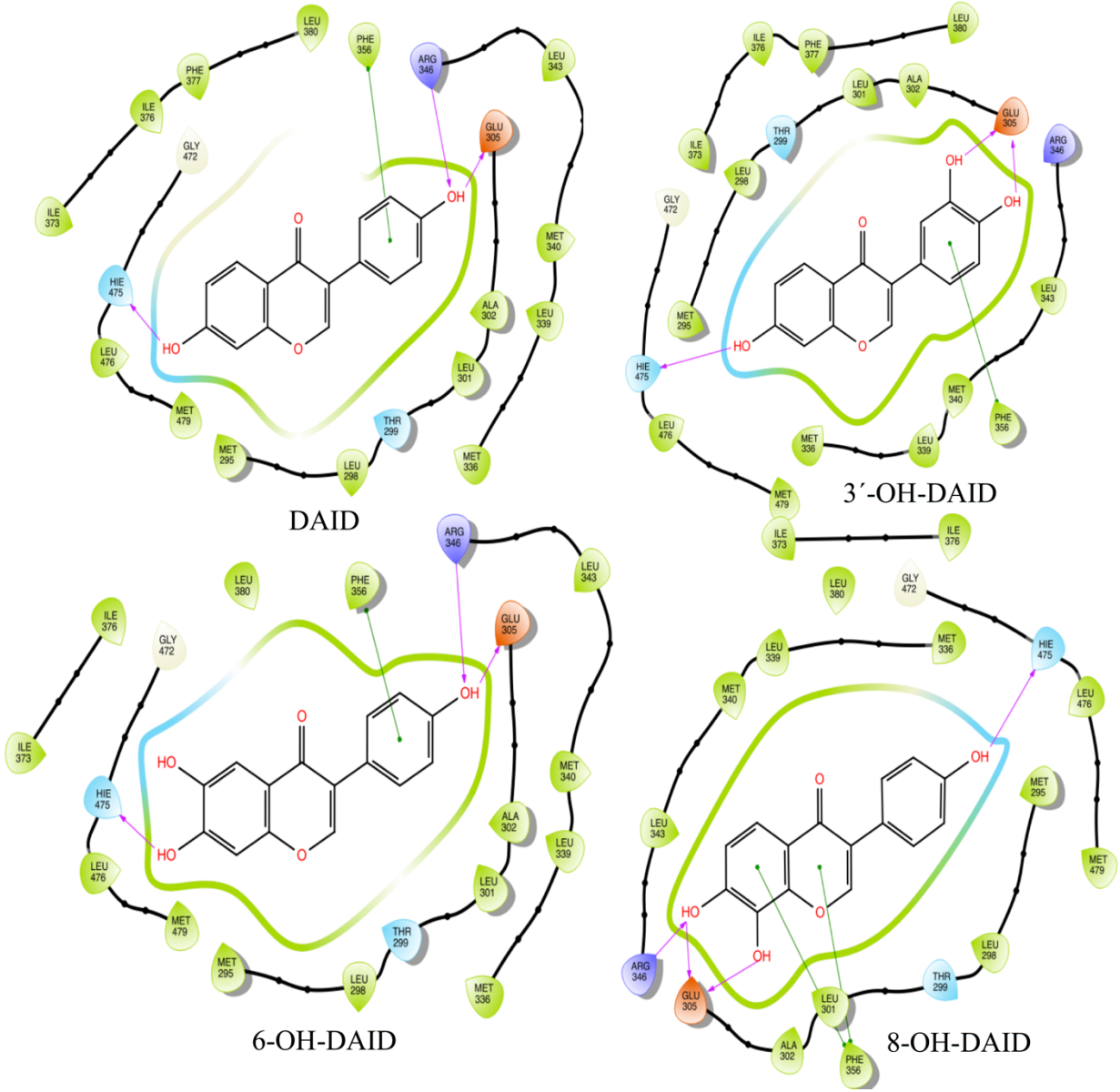


Figure 5.3: See the next page for legend.

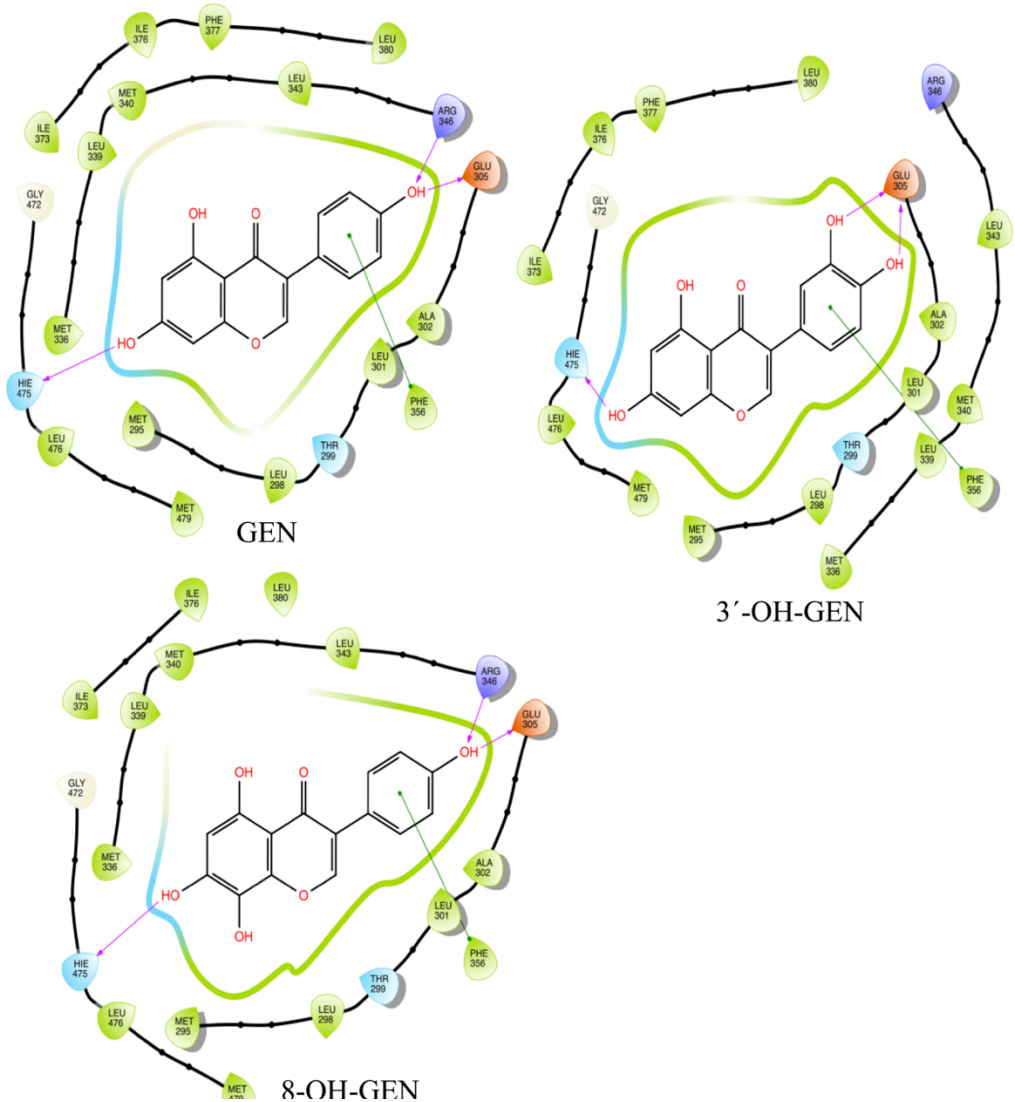
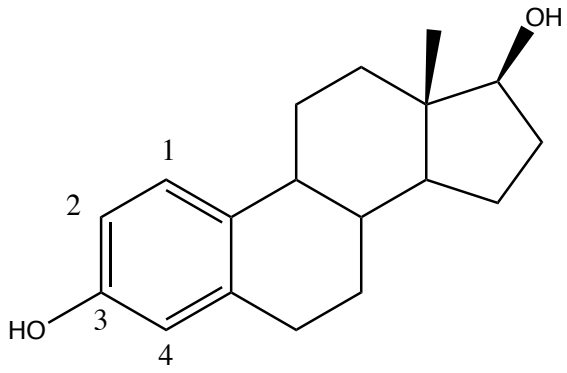


Figure 5.3: Isoflavones interaction with amino acid residues in ERβ visualised in Schrödinger. Purple arrows represent H-bonds; the direction of arrow denotes electron donor to electron acceptor in the H-bond. Different coloured residues represent amino acid properties: blue, positive charge; red, negative charge; cyan, non-charged polar; green, nonpolar. (NB: Schrödinger uses a non-standard amino acid abbreviation system the amino acid: HIE = Histidine).

Section 3.4.5, Chapter 3 introduced that, a hydrophobic ligand ‘prefers' to sit amongst hydrophobic residues in a hydrophobic binding cleft; conversely, the increased hydrophilic character could cause unfavourable interactions to a hydrophobic cleft and likely reduces ideal thermodynamic fit.[206] Table 5.1 shows that GEN, DAID, and their hydroxylated metabolites do not have remarkable different HER (rang from −5.38 to −5.68 kcal/mol), this likely result from the same hydrophobic regions (isoflavone’s molecule skeleton, Fig. 5.3) of these ligands of ERβ. However, the different arrangements hydroxyls still affect the HER. For example, compared with DAID, 8-OH-GEN has additional hydroxyls that cannot form any H-bonds with amino acid residues at the LBC because of their orientation (Fig. 5.3). The free hydroxyls of 8-OH-GEN (5, 8 -OH) are unfavorable to the hydrophobic binding pocket of ERβ, reducing 8-OH-GEN’s hydrophobicity and decreasing its binding energy (Table 5.1). This also could explain the similar HER of DAID (−5.68 kcal/mol) and 3′-OH-DAID (−5.64 kcal/mol), because the additional hydroxyl of 3′-OH-DAID forms a H-bond with LBC, which does not have unfavourable interaction with the LBC (Fig. 5.3). In addition, the extra polar substituent might influence the hydrophobicity of a ligand and interfere with ligand’s ERα hydrophobic interactions. This can be supported by previous study showing that both 4-OH-estradiol and 2-OH-estradiol have weaker RBA than E2 (Table 5.2).[52]

Table 5.2: The RBA of 4-hydroxyestradiol and 2-hydroxyestradiol with ERα, where the binding affinity of E2 equals 100.[52]



Compound	RBA
E2	100
4-hydroxyestradiol	13
2-hydroxyestradiol	7

This *in silico* study of isoflavones could be helpful in furtherly our understanding of the interactions between estrogens (include endogenous estrogens and xenoestrogens) and the LBC of ERβ. This indicates that the structural features of ligands could determine the presence and strength of their noncovalent interactions (e.g., H-bonds) with the targeted receptor, resulting in different binding energies and different binding affinities, and bioactivities, which in turn differentiates the ERβ-driven effects of individual isoflavones.

5.4.2 Effects of isoflavones on the proliferation of Caco-2 cells

Figure 5.4 shows that Caco-2 cell proliferation increased over 7 d of culture, and suggesting the log phase of the cell proliferation is from 3d to 4 d. However, the rate of proliferation is observed to slow by 5d; this is likely due to Caco-2 cells start to form a confluent monolayer.[272]

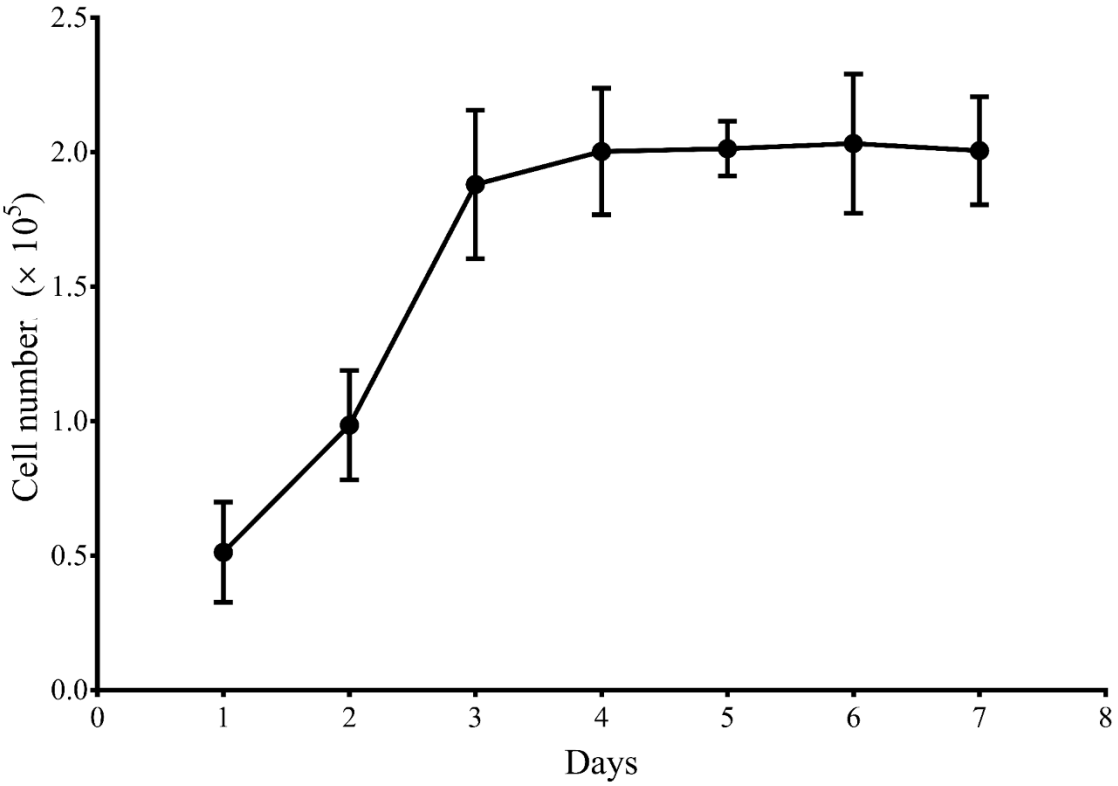


Figure 5.4: Caco-2 cell growth profile in culture; the values are mean ± SD (n=3) of the cell numbers are shown.

As discussed before, the proliferative effects of E2 and GEN on the Caco-2 cell line is ERβ mediated.[52, 270, 271] The *in silico* research studied isoflavones with different substitution (i.e., hydroxyls) patterns were predicted to dock with ERβ and gained similar DockingScore compared with GEN, this suggests that the seven selected isoflavones might interact with ERβ and likely have similar ERβ-driven bioactivity with GEN (i.e., increase the proliferation of Caco-2 cell).

The results of experiment studying Caco-2 cell proliferation show isoflavones have a dose dependent stimulatory effects on proliferation of Caco-2 cells, but the effects of isoflavones were significantly weaker than E2 (Fig. 5.5). Also, the comparison between the group of isoflavones (either GEN or 3′-OH-DAID) alone and an isoflavone (either GEN or 3′-OH-

DAID) combined with ICI 182,780 further supports that the effect of isoflavones on the proliferation of Caco-2 being ERβ-mediated (Fig. 5.6).

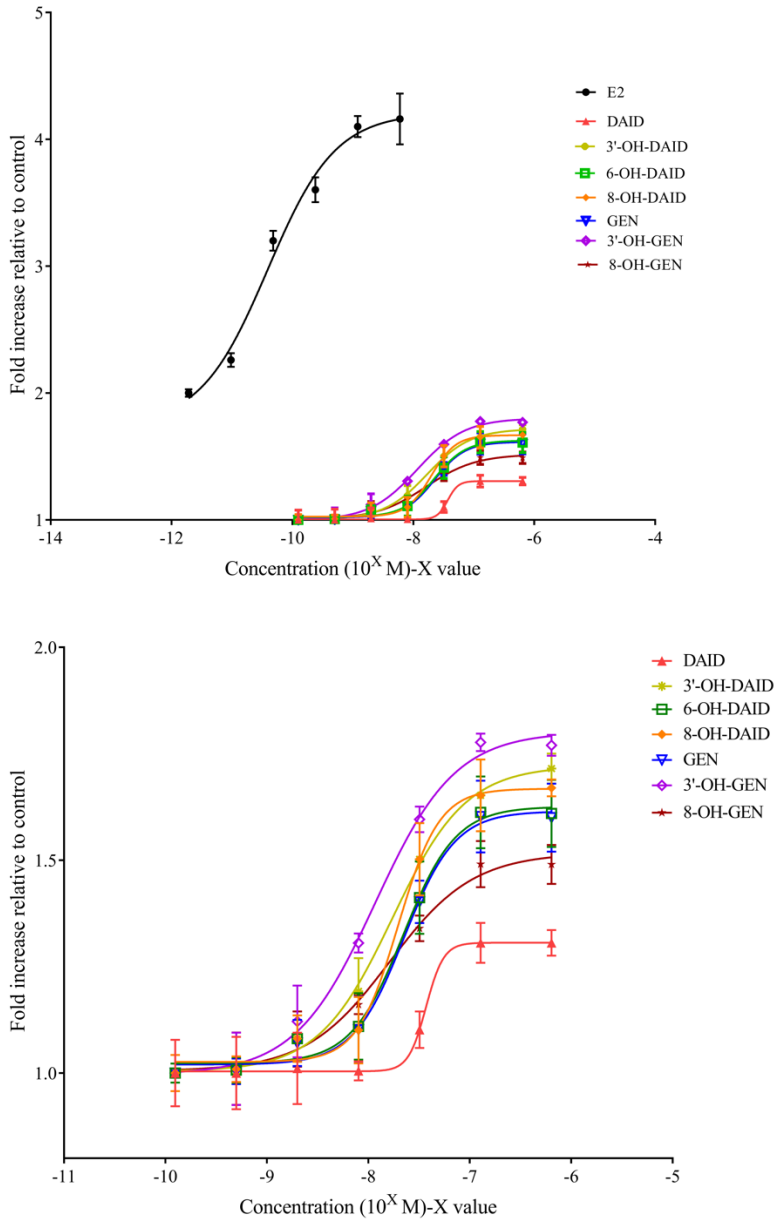


Figure 5.5: The effects of different exposure concentrations of E2 and isoflavones on the Caco-2 cell proliferation. The values are mean ± SD (n=3) of the cell numbers at a given concentration of a test isoflavone ratio to the blank.

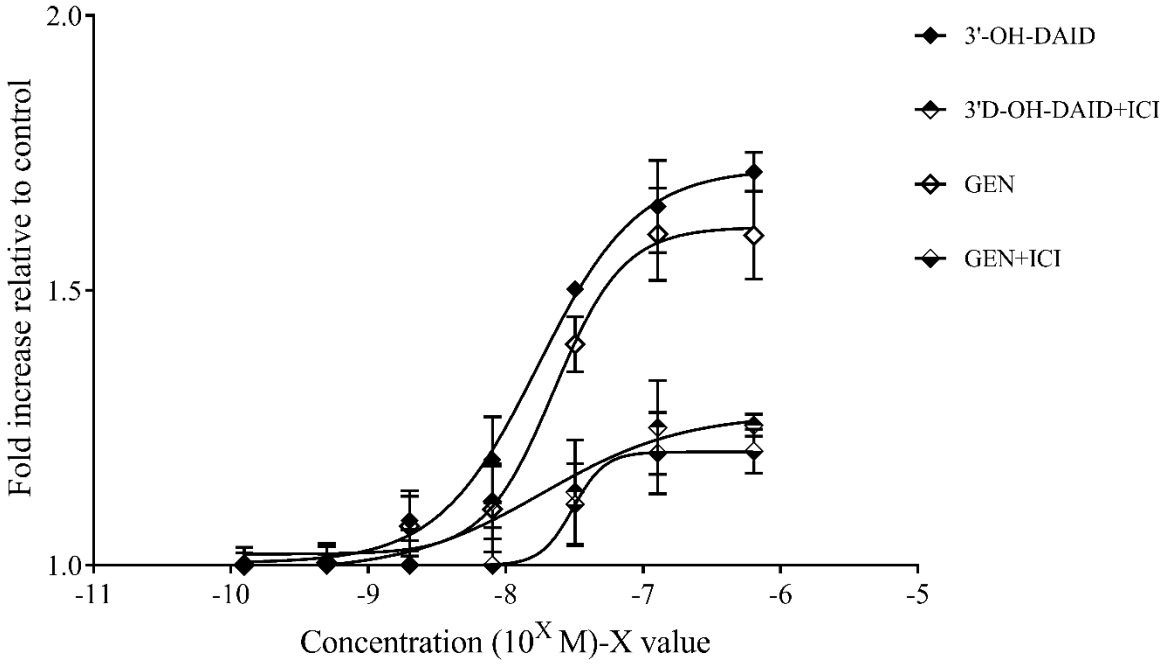


Figure 5.6: The effects of different exposure concentrations of 3′-OH-DAID and GEN on the Caco-2 cell proliferation. Caco-2 cells were grown in either 3′-OH-DAID or GEN in presence or absence of 1 μM ICI 182,780. The values are mean ± SD (n=3) of the cell numbers at a given concentration of a test isoflavone ratio to blank.

In this study, the effects of selected isoflavones on the proliferation of Caco-2 cell were determined. Table 5.3 shows EC_{50} and RAAs of the selected isoflavones, where E2 equals 1. The stimulatory activity of studied isoflavones (EC_{50} ranges from 3.64×10^{-7} M to 1.70×10^{-5} M) at least 10^{-3} times than E2 ($EC_{50} = 3.911 \times 10^{-11}$ M). The RAA values of the studied isoflavones showed a trend; hydroxylated metabolites have greater effects on the proliferation of Caco-2 cell than their parent compounds (i.e., GEN and DAID respectively). For example, 6-OH-DAID (1.77×10^{-3}) > DAID (6.12×10^{-5}) and 3′-OH-GEN (3.35×10^{-3}) > GEN (1.74×10^{-3}) (Table 5.3), this trend follows the predicated order of binding affinity between parent compounds and their hydroxylated metabolites in the *in silico* study shown in Table 5.1. However, 8-OH-GEN does not follow this tread is an exception; this compound was

predicted to have a greater binding energy than the parent compound GEN, but surprisedly showed a weaker activation on the proliferation of Caco-2 cell. Studied further in the *in silico*, the additional hydroxyl groups (Fig. 5.3) on 8-OH-GEN did not form H-bonds with amino acid residues at LBC, and is a unfavourable fitting to the hydrophobic region of the binding cleft; this might result in a low binding affinity and influence the receptor-driven bioactivity of 8-OH-GEN.

Table 5.3: EC_{50} and RAA values for isoflavones.

Compounds	**EC_{50} (M)**	**RAA**
E2	3.911×10^{-11}	1
3'-OH-GEN	1.168×10^{-8}	3.35×10^{-3}
3'-OH-DAID	1.698×10^{-8}	2.30×10^{-3}
8-OH-DAID	1.972×10^{-8}	1.98×10^{-3}
6-OH-DAID	2.212×10^{-8}	1.77×10^{-3}
GEN	2.246×10^{-8}	1.74×10^{-3}
DAID	6.386×10^{-7}	6.12×10^{-5}
8-OH-GEN	2.411×10^{-7}	1.62×10^{-4}

Binding affinity is the strength of the binding interaction between a single biomolecule (e.g., ERα) and its ligand.[246] Binding affinity is influenced by non-covalent intermolecular interactions (e.g., H-bonds, hydrophobic interactions) between the receptor and a ligand, which comprises free binding energy.[184] Results of the *in silico* study (Table 5.1) show that all hydroxylated metabolites have higher theoretical binding energy—DockingScores than their corresponding parent compounds; this predicts that these hydroxylated compounds may have a greater binding affinity with ERβ than their precursors. The additional hydroxyls might form extra H-bonds with specific adjacent amino acid residues in the LBC of ERβ (e.g., His 475) (Fig. 5.3); thus, this might increases the binding energy and binding affinity with ERβ (e.g., DockingScore of DAID = −11.03 kcal/mol, DockingScore of 3'-OH-DAID = −11.56 kcal/mol) (Table 5.1).

The Caco-2 cell experiment showed that the hydroxylated isoflavone metabolites have a greater effect on the proliferation of Caco-2 cell than their less hydroxyl parent compounds. For example, the RAA of 3'-OH-DAID is 2.27×10^{-5} which is higher than DAID (its RRA equals 1.48×10^{-6}. This indicates that the hydroxylation of isoflavone contributes to their ERβ-mediated activities. In addition, based on the same ligand–receptor system, previous research showed biochanin A with an additional hydroxyl is more estrogenic than FOR (Fig. 5.7).[223] Again, previous research reports that in an inhibitor-enzyme system, which requires specific binding characteristics of substrates for interacting with the corresponding enzyme,[276] kaempferol (a tetrahydroxy-flavonol) is a stronger inhibitor of XO than galangin (a trihydroxyl-flavonol) (Fig. 5.7).[218] Interestingly, in the *silico study*, 8-OH-GEN was predicted to have higher binding affinity with ERβ (Table 5.1) but resulted in less Caco-2 cell proliferation than GEN (Table 5.3); this anomaly might be explained by the two free hydroxyls of 8-OH-GEN (Fig. 5.3) which might not form H-bonds with any amino acid residues in the LBC because there are no free potential amino acid residues in the vicinity of the docked ligand (Fig. 5.3). These free hydroxyls are not favourable to the hydrophobic binding pocket of ERβ and might resulting in a decrease in the ligand's hydrophobicity, thus leading a reduction of the binding affinity of the ligand with ERβ. Similarly, published result

shows kaempferol, which has one more hydroxyl (Fig. 5.7), has a weaker estrogenic potency than galangin.[190]

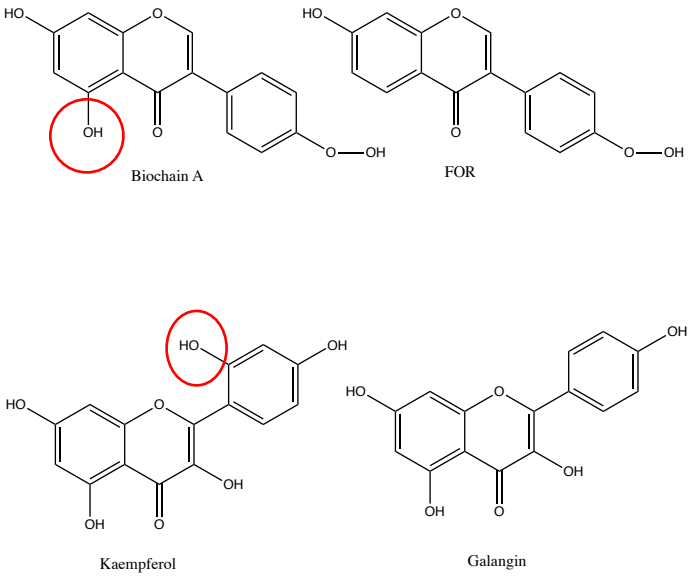


Figure 5.7: Structures of FOR, biochanin A, kaempferol, and galanin. The extra hydroxyl of biochain A and kaempferol compared with FOR or galangin correspondingly are highlighted by a red circle.

5.4.3 The influence of intestinal phase II metabolism on the ERβ-driven activity of selected isoflavone in Caco-2 culture

Isoflavones (e.g., GEN) have been reported to show higher binding affinity to ERβ than ERα.[52] For example, the RBA of GEN is 5 with ERα and 36 with ERβ, where the binding affinity of E2 equals 100 with both ERα and ERβ.[52] Likewise, the results of YES assays show that the EC_{50} of GEN is 9.0×10^{-7} M with ERα and 4.2×10^{-9} M with ERβ. Interestingly, the Caco-2 cell study does not show correspondingly strong effect of isoflavones on the ERβ-mediated cell proliferation (e.g., the EC_{50} of GEN is 2.246×10^{-8} M). As discussed before (***Section 5.1.3***), the Caco-2 cell line is a continuous cell line of

heterogeneous human epithelial colorectal adenocarcinoma cells and is able to carry out phase II metabolism, during which, xenobiotics (e.g., isoflavones) might be conjugated with glucuronic acid and be converted into non-bioactive conjugates.[269] The intestinal phase II metabolism might thus result in a reduction of the level of functional form of isoflavones, this could influence the interplay of such isoflavones with macromolecules (e.g., ERβ), which, might in in turn, affect macromolecule-mediated bioactivities of isoflavones in different tissues.

In this study, Caco-2 cells were pre-treated with 37.5 μM GA for 12 h to inhibit the UGDH activity, then incubated with either GEN or 8-OH-GEN (6.4×10^{-2} μM) for another 72 h. Previous research showed isoflavones (e.g., GEN) can be conjugated by glucuronosyltransferase with uridine diphosphate glucuronic acid (UDP-glucuronic acid), which is produced with UDP-glucose dehydrogenase (UGDH).[269, 277] GA is a non-competitive inhibitor of UGDH activity and it can reduce the production of UDP-glucuronic acid at post-translational level, which, in turn, would interfere with glucuronidation of isoflavones.[278] Figure 5.8 shows that the Caco-2 incubation with the presence of GA did not show any significant reduction in cell proliferation compared with the blank. In addition, GA pre-treatment resulted in enhancement effects on both GEN and 8-OH-GEN induced proliferation of the Caco-2 cells ($p < 0.05$); this is likely due to the inference of GA with the conjugation of isoflavones increasing the amount of functional forms for interacting with ERβ. This could explain that the why isoflavones showed higher binding affinity with ERβ than with ERα,[210] but did not exhibit a correspondingly greater ERβ-driven proliferative effect on Caco-2 cell.

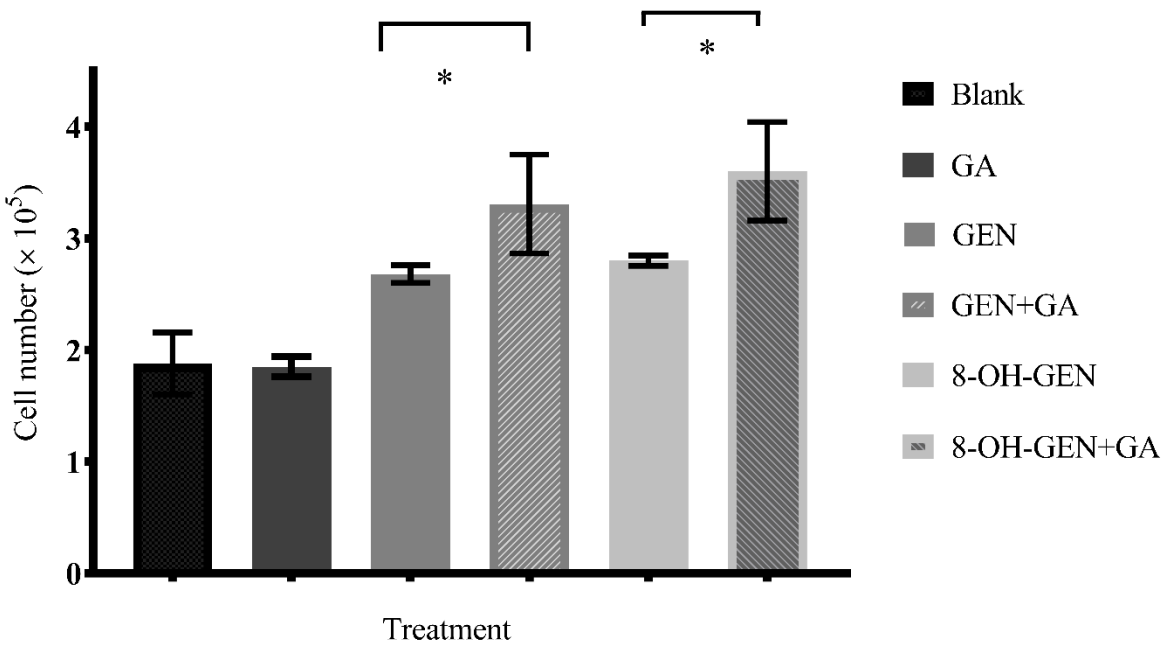


Figure 5.8: Proliferation of Caco-2 cells following exposure to GEN or 8-OH-GEN in the presence or absence of GA. Caco-2 cells were grown in media containing GEN or 8-OH-GEN (6.4×10^{-2} μM) in the presence or absence of GA 37.5 μM. *Significant differences between cell number in isoflavone alone incubation compared with incubation containing isoflavone and GA were calculated by one-way ANOVA (*: $p < 0.05$).

In this study, the non-competitive inhibitor of UGDH—GA was used to inhibit the activity of UGDH; this would decrease the biosynthesis of UDP glucuronic acid which likely provides glucuronic acid for the conjugation of isoflavones.[269] This bio-action will lead to the conversion of isoflavones to non-bioactive conjugates (Fig. 5.9). Thus, the GA pre-treatment of Caco-2 cells likely increases the amount of the functional form (i.e., bioactive aglycone) of the isoflavone in the cells; thus, promoting their ERβ-driven effects on Caco-2 cell proliferation.

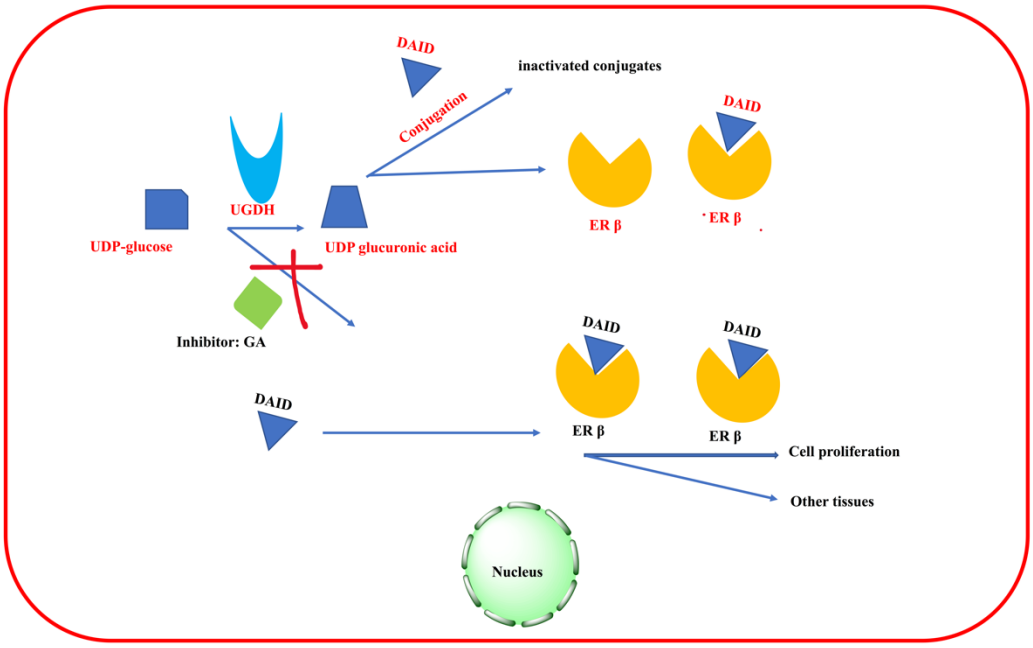


Figure 5.9: The effects of UDP conjugation on isoflavones (e.g., DAID) estrogenic bioactivity in Caco-2 cells. Left of Schematic: UDP glucuronic acid is produced from UDP-glucose catalysed by UGDH, then UDP glucuronic acid conjugates with DAID to yield a non-bioactive conjugate of DAID. The conjugation decreased the amount of the functional form (i.e., aglycone) of DAID resulting in less DAID for interacting with ERβ. An UGDH inhibitor—GA influences the production of UDP glucuronic acid, this results in accumulation more DAID which would interact with ERβ or be transferred to other tissues.

5.5 Conclusions

In this chapter, the Schrödinger platform was used to determine the theoretical binding energy represented by the DockingScore and to predict the order of ERβ binding affinity of GEN, DAID and their hydroxylated metabolites. This *in silico* study indicated the hydroxyls patterns likely determines the H-bond interactions and hydrophobic interactions of isoflavones with amino acid residues in the LBC of ERβ; this, in turn, might result in different binding energies and thus different estrogenicities. The *in silico* study indicates a trend: more hydroxyls form more H-bonds leading to higher receptor binding energy. For example, the DockingScore of 6-OH-DAID (−11.88 kcal/mol) is higher than DAID (−11.03 kcal/mol). However, the extra hydroxyl, which cannot form H-bond with the amino acid residues in the LBC, in theory decreases the hydrophobicity of the ligand causing unfavourable interaction with the LBC, thus reducing the ligand's binding energy; for example, the DockingScore of 8-OH-GEN (−11.64 kcal/mol) is lower than 3′-OH-GEN (−11.72 kcal/mol). This theoretical approach to studying ligand/ ERβ interaction gives important insight into the intimate biological chemistry, whether these *in silico* prediction translate to *in vivo* studies was studied of ERβ-expressing Caco-2 cells.

Results of Caco-2 cell proliferation experiments show that the proliferative effects of isoflavones on Caco-2 cell line were dose-dependent. Hydroxylated metabolites of GEN and DAID show greater proliferative effects on Caco-2 cells, compared with their less hydroxylated parent compounds. However, the exception to this trend is 8-OH-GEN, which has a significantly weaker proliferative effect than GEN. This might be due to the additional hydroxyl groups of 8-OH-GEN (Fig. 5.3); because there are no free potential hydrogen binding amino acid residues in the vicinity of docked ligand. In addition, the extra hydroxyl groups would decrease the hydrophobicity of the ligand. Interestingly, isoflavones have a higher binding affinity with ERβ than with ERα,[210] and a higher relative potency in the YES assay with ERβ than with ERα.[279] However, such isoflavones did not show correspondingly

greater effects in the Caco-2 cell line (which expresses predominantly ERβ). In this study, GA's inhibition of UGDH might influence the conjugation of isoflavones and increase the amount of isoflavone aglycones in cells; this, in turn might promote the proliferative effects of isoflavones on Caco-2 cells. This finding might be significant in a health context because GA is present in many fruits (e.g., strawberry, banana) and in tea.[278] This suggests that in a 'food combination' situation, it is feasible that a complex interplay between GA inhibition, isoflavone deactivation in conjecture with metabolic (hydroxylation) activation of isoflavones could significantly increase and prolong their ER-mediated biological effects. This might play a role in determine the functionality of isoflavone-containing foods.

Chapter 6: Structure bioavailability relationships of isoflavones and their effects on modelled gut bacterial populations

6 Chapter 6 – Structure bioavailability relationships of isoflavones and their effects on gut bacterial populations

6.1 Introduction

6.1.1 The metabolism of isoflavones

As discussed before (***Section 1.3.4 Chapter 1***), soy isoflavones (i.e., GEN and DAID) are phytoestrogens, which can occupy and activate ERs, and might cause corresponding estrogenic bioactivity *in vivo*.[280 281 70] Soy isoflavone is the main dietary phytoestrogen because of their high concentrations in soy and the huge consumption of soy-based foods.[280 281] The metabolism of isoflavones is complex.[282] The two major isoflavones, GEN and DAID, are present in raw soy as their β-D-glycosides, namely genistin and daidzin (Table 1.3). These glycosides are biologically inactive.[122] Once ingested, isoflavone glycosides are hydrolysed by bacterial β-glucosidases in the intestine, resulting in conversion to their corresponding bioactive aglycones (i.e., GEN and DAID, respectively).[282] Interestingly, the β-glucosidases gene is well present in Bacteriodete*s* and Firmicutes in the human intestine; this suggests the individual differences of these two type of bacteria may vary the hydrolysis of isoflavones glycosides and resulting different amount of aglycones.[282] In addition, only the aglycones are absorbed in the intestine and are, therefore biologically active. Consequently, the individual different compositions of gut bacteria could affect the hydrolysis of glucosides isoflavones and influence the functionality of isoflavones.[229] Moreover, aglycones might be further converted to different estrogenic metabolites. For example, DAID can be metabolised to more estrogenic *S*-equol (Fig. 3.3), and GEN would be converted into less estrogenic dihydrogenistein by intestinal bacteria (Fig. 3.3).[283] All parent compounds and metabolites of isoflavones (e.g., GEN and DAID) have been detected in the blood and urine of humans and animals.[284] The metabolic pathways of isoflavones have individual variations; [285] for example, only 30% of humans can metabolize DAID into *S*-equol, which has been associated

with individual difference in gut bacterial composition;[229] this could individually differentiate the *in vivo* bioactivity (e.g., estrogenic regulation) of DAID. Recently, it has been reported that 'equol producers' have a lower prevalence of obesity than 'non-equol producers;[286] this might be linked to the estrogenic regulation of fatty acid distribution and energy homeostasis.[287]

6.1.2 Gut bacteria and human health

The human gut hosts billions of bacteria; the two major phyla, that make up 90% of the gut commensal microbiota are Bacteroidetes and Firmicutes, the most abundant genera of which are *Bacteroides* and *Faecalibacterium*.[288] The phylum Actinobacteria is much less abundant, but it includes *Bifidobacterium*, which is a genus used in food supplements for delivering probiotic benefits to the host.[289] The diversity of host-bacteria interactions, which could be influenced by diet or host health situations, plays a fundamental role in the well-being of the host *via* a wide range of pathways.[288] For example, gut bacterial communities are suggested to contribute to the development of insulin resistance.[53] Previous research indicates different diets affect the population of gut bacteria,[290] the study using mice fed a high-fat diet shows an improvement in glucose insulin homeostasis and attenuation of weight gain when mice were given *Lactobacillus* and *Bifidobacterium* probiotics, compared to mice given a normal diet without probiotics.[290] Another example is nonalcoholic fatty liver disease, the changing of gut microbiota has been observed in patients. Bacteroidetes (including *Prevotllaceae*) and Saccharibacteria were increased, but the main of phylum of Firmicutes including *Lactobacillus* were decreased.[53] *F. prausnitzii* is one of the most abundant and important commensal bacteria.[291, 292] In healthy adults, *F. prausnitzii* represents more than 5% of the bacteria in the intestine, and can produce butyrate and other short-chain fatty acids (SCFAs) through the fermentation of dietary fibre; these SCFAs might benefit colonocytes by increasing energy production and promoting cell proliferation, in turn, it has been suggested that they might protect against gut diseases (e.g., colon cancer).[291] In addition, published data show lower than usual concentrations of *F. prausnitzii* in the intestines are associated with

Crohn's disease, obesity, asthma and major depressive disorders, while higher than usual concentrations have been associated with psoriasis.[291]

Obesity is a physiological state that has emerged as a major health concern in populations, and this is closely linked to the gut bacteria.[293] In animal model studies of obesity, the increased efficiency of energy harvest due to an increase in the ratio between the concentration of Firmicutes/Bacteriodetes (F/B) has been implicated in the aetiology of obesity in mice.[294] The same change of F/B was observed humans on weight-reduction diets; although another study showed that in obese individuals, a decrease in Bacteroidetes was accompanied by an increase in Actinobacterium rather than Firmicutes.[294,295] These observations imply the important role of Bacterioidetes which has been linked to their sphingolipids production modulated lipid homeostasis.[296] In addition, published data show the consumption of a high-fat diet in both animals and humans can result in both changes in the composition of gut bacteria and resulting an increase intestinal permeability;[269] this might result in an increase in endotoxin concentrations (e.g., lipopolysaccharide). The lipopolysaccharide plays a central role in maintaining the integrity and functionality of the outer membrane of the Gram negative cell envelope,[297] and thus, contribute to the low-grade inflammation, insulin resistance, adipocyte hyperplasia, and decreased β-cell function.[298]

Section 1.5.2, Chapter 1 introduced two types of biotransformation products of DAID and GEN, namely OH-DAIDs and OH-GENs (Fig. 1.12), which have been detected in various fermented soybean foods (e.g. miso and soy sauce).[157, 252] These metabolites inherit the same structural skeleton from their parent compounds, but with different hydroxyl patterns (Fig. 1.12).[157, 251, 252] The absorption and metabolism of xenobiotics (e.g., GEN) have been widely studied in Caco-2 cell monolayer system which mimic human intestinal mucosa.[1, 277] Previous research shows such xenobiotics can be transferred through the Caco-2 cell monolayer and conjugated in the cells by phase II metabolism; this determines the bioavailability, and in turn, influences the functionality of such xenobiotics.[299]

6.2 Research Objectives

- Use an *in vitro* gut fermentation model to investigate the effects of isoflavones on human gut bacteria.

- Use a Caco-2 monolayer transportation model gut system to study the absorption and metabolism of GEN and DAID and their corresponding hydroxylated metabolites.

6.3 Experiments

These studies were carried out in collaboration with Dr. Shanthi Parkar, and the experiments were carried out in her laboratory (Plant and Food Research, Palmerston North).

6.3.1 *In vitro* gut fermentation.

The feces samples used as inoculates were obtained from 5 human volunteers with no *a priori* selection criteria beyond self-assessed good health and no intake of antibiotics for at least 3 months prior to the study (Central Health and Disabilities Ethics Committee Approval # 13/CEN/144). Pooling of the feces samples produced a standard with a diverse range of bacteria from different donors and avoided the inter-individual differences of bacterial compositions. These were processed under anaerobic conditions and stored as 20% v/v fecal slurries in 10% v/v glycerol buffered saline at −80 °C.[300]

The preparation of WCA broth and experimental regents were described in the ***Chapter 2***. The WCA broth (50 mL) containing each individual isoflavones (5 µM) or inulin (1 mg/mL) was incubated with human fecal slurry (1% v/v, final concentration). Inulin, which is a prebiotic fibre, is used as the positive control in this study. The media and buffers for the gut fermentations were left in an anaerobic chamber maintained at 5% CO_2, 10% H_2 and 85% N_2 for at least 24 h before the start of the isoflavones exposures. Fermentation was carried out in the anaerobic chamber at 37 ℃ rotating at 70 rpm. Aliquots (1 mL) of the fermented mixture were collected at 1, 2 and 20 h, and centrifuged at 13,000 × g at 4°C for 1 min. The pellets were separated and stored at −80°C prior to quantification of bacteria. The negative controls were WCA alone and WCA broth inoculated with the fecal microbiota.

6.3.2 Real-time PCR quantification of bacteria

The total DNA was isolated from each fermentation aliquot using the ZR Faecal DNA kit following the manufacturer's instructions. The extracted DNA then were ready for bacterial quantification by PCR. The concentrations of two phyla Bacteroidetes and Firmicutes, the concentrations of a specific species of Firmicutes—*F. prausnitzii*, and *Bifidobacterium* spp, in each sample were quantified using previously published real time PCR protocols.[170, 301, 302] Real-time PCR (RT-PCR) was performed with the Rotor-Gene 6000 machine, in a final volume of 10 µL containing 5 µL of LightCycler® 480 SYBR Green I Master mix, 3.8 µL Milli-Q water, 0.1 µL of each primer (10 µM) and 1 µL of DNA (1 in 10 diluted sample). The reaction conditions were 95℃ for 5 min followed by 35 cycles at 94 ℃ for 15 s, and then annealing at conditions suitable for each primer set (see Table S2, Appendix C), an extension at 72°C for 60 s, followed by a melt cycle to determine the specificity of the amplification product. The reference bacterial strains and the primers used to construct the standard curves for and quantify the bacteria are given in Table S2 Appendix C. Bacterial quantitation was done using Rotor-gene 6000 series software 1.7, and expressed as $\log_{10}$ colony forming unit/mL/fermenta (cfu/mL).

6.3.3 Cell culture

The Caco-2 transportation experiment of isoflavones was carried out in collaboration with Associate Professor Jacqui Keenan, and carried out in her laboratory (Christchurch School of Medicine, University of Otago).

The preparations of cell culture media and experimental regents were described in ***Chapter 2***. Cell culture methods were adapted from Budd GR *et al*.[272] Caco-2 (ATCC HTB-37) cell line used in this study was derived from a human colon adenocarcinoma and is widely used to model differentiating gut enterocytes.[272, 271] The cells were routinely cultured in the pre-prepared MEM with Earle's Salt and L-glutamine, supplemented with 10% (v/v) heat-

inactivated FBS. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.[272]

6.3.4 Isoflavone transport experiments in the Caco-2 gut model system

Caco-2 cells were seeded into a 24-well transwell insert coated with type-I collagen at a density of 5×10^4 cells/well to form Caco-2 monolayers. The medium was replaced every 3 d, and the monolayers were cultured to differentiate for 24 d post seeding. Before the experiment, the integrity of the cell layer and the development of the tight junctions between cells were monitored by measuring the transepithelial electrical resistance (TEER) of filter-grown cell monolayers with Millicell Voltohmmeter. Only a monolayer with the TEER value >260 **Ω** hm cm^2 was applied for the isoflavone transport experiments.[303]

The cell monolayers were gently washed three times with PBS (pH 7.4, 37°C). Cell monolayers were then incubated at 37°C for 30 min in the PBS. To measure the apical-to-basolateral (AP-BL) transport, 100 µL of PBS containing isoflavones (final concentration 5 µM) were added to the AP side of the transwell inserts, and 600 µL of culture medium was added to the BL chamber. The plate was incubated at 37°C for 2 h.

6.3.5 Samples collection

The isoflavone extraction method was adapted from our previous research.[199] Culture media from AP and BL media were directly collected for extraction. The Caco-2 cells were washed with PBS (37°C) three times, 200 µL RIPA buffer was added, then incubated on ice for 20 min with occasional mixing to ensure cell detachment and lysis. Cell lysates were collected then centrifuged at 14000 × *g* for 30 min at 4°C to remove remaining cell debris. The supernatants (containing isoflavones) were collected for extraction and analysis by HPLC.

6.3.6 Extraction of samples of Caco-2 transportation experiment

Samples (2 mL) prepared from previous section were extracted by vortex mixing with ethyl acetate (3 times volume of sample) in triplicate. The upper ethyl acetate layer of each sample transferred into a new test tube and evaporated under a stream of N_2 gas (40 °C), this was designed Group I. The aqueous (bottom) layer from each extracted sample was treated with glucuronidase and sulfatase to realise aglycones of the isoflavones, then were shaken at 37 °C for 2 h,[269, 277, 304] The incubate was extracted with ethyl acetate, the ethyl acetate layer was collected and evaporated as above.; this was designed Group II. Residues from Group I and II were dissolved in HPLC-grade acetonitrile (1 mL) containing 10 % (v/v) DMSO and filtered through a 0.45 µm non-sterile syringe filter into a 2 mL glass HPLC vial and then analysed by HPLC.

6.3.7 HPLC analysis

The HPLC method was same as from our previous research.[199] The HPLC set-up comprised Dionex hardware (sample injector ASI-100, thermostatic column compartment TCC-100;) with a Phenomenex C_{18} Prodigy 5µm ODS3 100 A 250 mm × 4.6 mm column with a C_{18} SecurityGuard column and an ultraviolet/visible detector; UV detection was at 254 nm. The HPLC mobile phases consisted of solvent A: Milli-Q water containing 0.05% (v/v) trifluoroacetic acid; solvent B: acetonitrile. A solvent gradient was run as follows: 0–25 min, solvent B linearly increased from 5% to 50%, remaining at 50% for 5 min; then solvent B decreased from 50% to 5% from 30 to 35 min. Correspondingly, solvent A linearly decreased from 95% to 50% over 0–25 min, remaining at 50% for 5 min; then solvent A increased from 50% to 90% over the final 5 min of the run. The injection volume was 20 µL, the flow rate was 1 mL/min, and running time was 35 min.

The calibration curves (Peak area vs concentration) for selected isoflavones (Table 5.1) all have good linear regression with R^2 values in the range of 0.98–0.99 (see Fig. S2, Appendix

C). The linear nature of the calibration graphs allows for the reliable calculation of concentrations of selected isoflavones in the range of 0.02–4 μM.

6.3.8 Caco-2 Isoflavone transport experiment: data analysis

The rates of transport (V_t) of each isoflavone were obtained using the concentration of transported isoflavones (dC) (from AP to BL) divide 2 h (t) (Eq. 1). The apparent permeability coefficient (P_{app})[303] across a cellular membrane was calculated using the rate of transport divided by the surface area (A) (0.33 cm^2) of the Caco-2 monolayer and the initial concentration, C_0 (5 μM) of these compounds (Eq. 2).[303] Calculation was carried out as follow.

$$V_t = dC/t; \quad \text{(Eq. 1)}$$

dC = change in concentration of isoflavone

$$P_{app} = V_t/AC_0 \quad \text{(Eq. 2)}$$

Raw data from the Caco-2 monolayer transportations of isoflavones were analysed using Microsoft Excel®. Mean ± SD was calculated. Significant differences were calculated by one-way ANOVA using GraphPad Prism (Version 7).

6.4 Results and Discussion

6.4.1 Effects of isoflavones on different bacterial populations

Previous research indicates that a bi-directional interaction exists between isoflavones and gut bacteria, this interaction is associated with host metabolic rate, body weight, and adiposity.[66, 286] On the one hand, isoflavones can be metabolised by the gut bacteria; the glycoside forms of isoflavones (e.g., daidzin) are hydrolysed releasing their aglycones (functional forms). In addition, it is possible that aglycones are converted into more estrogenic compounds (e.g., DAID is converted to *S*-equol).[229] However, the effects of isoflavones on the composition and population of human gut bacteria are not well understood. One limited example is GEN which has been reported to significantly alter the compositions of the fecal bacterial community in postmenopausal women by increasing the concentration of *Bifidobacterium*s spp, while decreasing Clostridiaceae.[283]

In this chapter, two phyla Bacteroidetes and Firmicutes, a specific species of Firmicutes—*F. prausnitzii*, and *Bifidobacterium* spp, were quantified. Figure 6.1 shows the effects of the selected isoflavones on the concentration of *Bifidobacterium* spp in the *in vitro* fermentation system; the positive control group (incubated with inulin), continued increasing the concentration of *Bifidobacterium* spp. from 6.21 $\log_{10}$ cfu/mL to 7.48 $\log_{10}$ cfu/mL over 20 h. Over the 2 h incubation, except 6-OH-DAID, all other isoflavones have significantly greater ($p < 0.03$) proliferative activity on the concentration of *Bifidobacterium* ranging from 6.87 $\log_{10}$ cfu/mL (8-OH-GEN) to 7.08 $\log_{10}$ cfu/mL (3′-OH-DAID) compared with the inulin control (6.24 $\log_{10}$ cfu/mL), but GEN and DAID do not have any significant difference compared with their corresponding hydroxylated metabolites (Fig. 6.1). Following 2 h incubate, *Bifidobacterium* decreased in all of the isoflavone exposure groups.

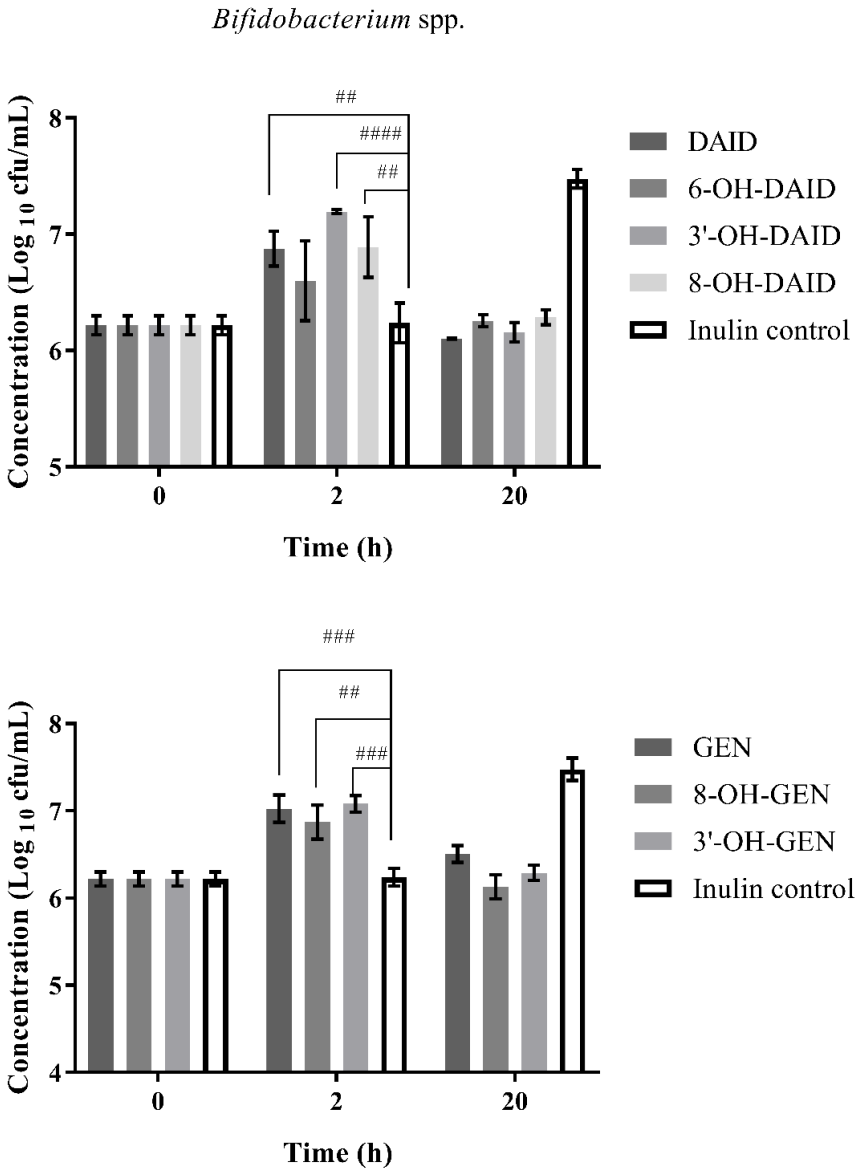


Figure 6.1: Effects of isoflavones on the concentration of *Bifidobacterium* spp.. Mean ± SD are shown. # Significant difference in the concentration of *Bifidobacterium* spp. (##: $p<0.03$, ###: $p<0.01$, ####: $p<0.001$), which was the comparison of each isoflavone with inulin control calculated by one-way ANOVA.

The *in vitro* gut fermentation experiment shows isoflavones (5 μM) affect the concentration of Firmicutes and Bacteriodetes over the 20 h incubation (Table S3, Appendix D), and the

F/B were calculated. Briefly, isoflavones decreased the concentration of Firmicutes and increased the concentration of Bacteriodetes, thus, decreasing F/B. The inulin control decreased the F/B over the 20 h incubation, while isoflavones resulted in a rapid decline in F/B over 2 h, and all of the studied isoflavones showed stronger effects on F/B (rang from 1.36 to 1.54) than the inulin control (1.56) in 2 h (Table 6.1); 3′-OH-DAID and 3′-OH-GEN are the most effective compounds at reducing the F/B compared with other isoflavones (Table 6.1).

Table 6.1: The effects of isoflavones on the F/B ratio.

Compound	Incubation time (h)	F/B
DAID	0	1.59
	2	1.43
	20	1.49
6-OH-DAID	0	1.59
	2	1.43
	20	1.53
3′-OH-DAID	0	1.59
	2	1.37
	20	1.45
8-OH-DAID	0	1.59
	2	1.54
	20	1.58
Inulin control	0	1.59
	2	1.56
	20	1.49
GEN	0	1.59
	2	1.40
	20	1.55

8-OH-GEN	0	1.59
	2	1.54
	20	1.85
3′-OH-GEN	0	1.59
	2	1.37
	20	1.54

The effects of the studied isoflavones on the concentration of *F. prausnitzii* in the 20 h anaerobic fermentation are shown in Figure 6.2. Over 2 h incubation, isoflavones increased the concentration of *F. prausnitzii;* this ranged from 5.38 $\log_{10}$ cfu/mL (3′-OH-DAID) to 5.82 $\log_{10}$ cfu/mL (GEN). In addition, only 6-OH-GEN has significantly greater effect on the the concentration of *F. prausnitzii* compared with the inulin control (5.74 $\log_{10}$ cfu/mL), and DAID exhibits significant inhibition compared with its hydroxylated compound 8-OH-DAID. However, the hydroxylated GENs have a similar effect on the concentration of *F. prausnitzii* as the parent compound, GEN (Fig. 6.2).

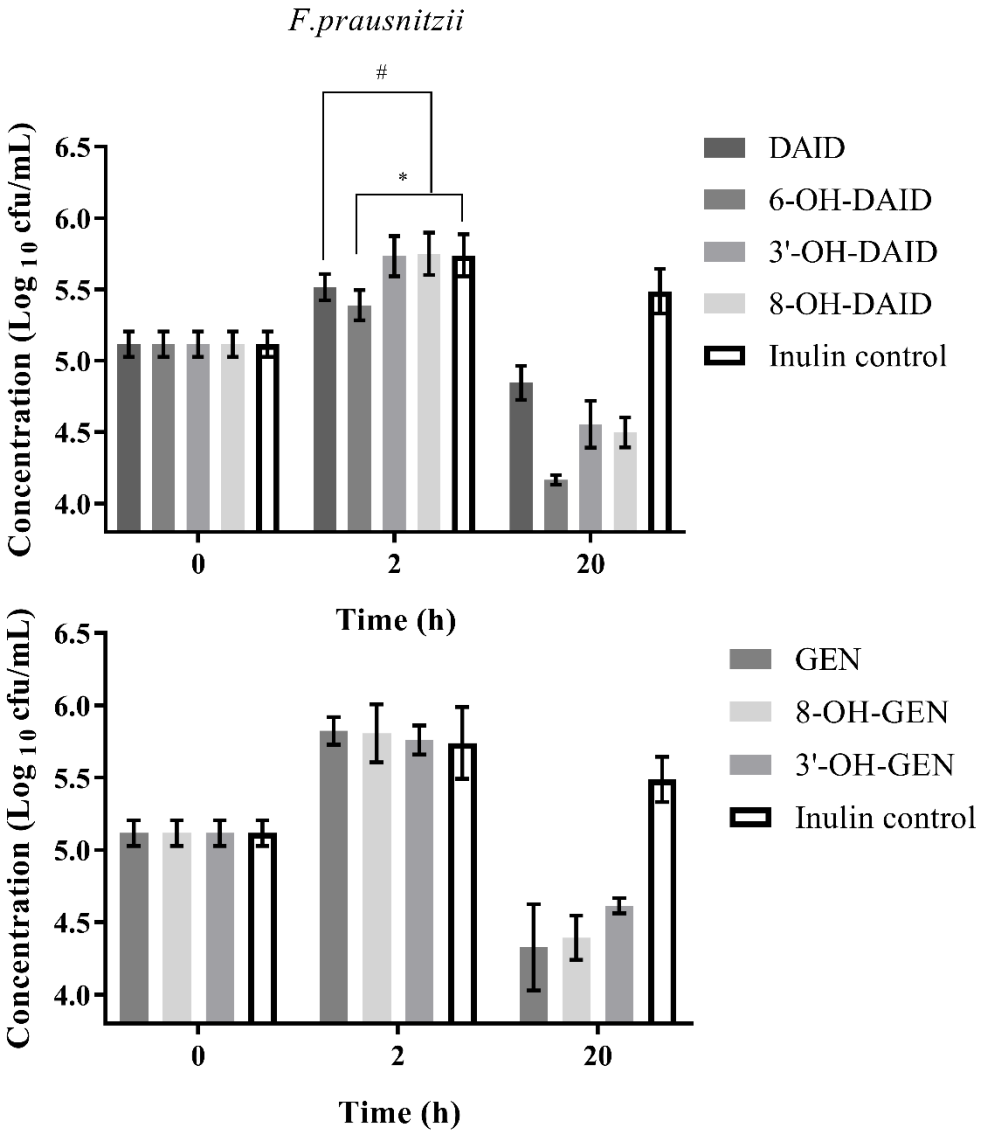


Figure 6.2: Effects of isoflavones on the concentration of *F. prausnitzii*. Mean ± SD are shown. *Significant difference in the concentration of *F. prausnitzii* (*: $p<0.05$) which was comparison of each isoflavone with inulin control calculated by one-way ANOVA. #Significant difference in the concentration of *F. prausnitzii* (*: $p<0.05$) which was comparison of each OH-DAID with DAID calculated by one-way ANOVA.

In a human health context, *Bifidobacterium* is one of the principal probiotic members in the gut, this genus of bacteria is used as a conventional treatment for ulcerative colitis and has

been associated with improved rates of remission and improved maintenance of remission.[305] Other beneficial gut commensal bacteria have been identified recently, such as the butyrate-producer-*F. prausnitzii*, which decreases in inflammatory bowel disease and is particularly associated with the pathophysiology of Crohn's disease.[306] The F/B has been shown to have some health implications and suggested as a potential parameter for the assessment of gut health.[307] For example, the F/B changes with age, for infants (0.4), adult (10.9) and elderly individuals (0.6), respectively. In addition, a higher F/B has been associated with obesity and type 2 diabetes, which might due to their important role in energy regulation *via* modulating the recirculation of estrogens.[308] In this study, isoflavones show different effects on the bacteria studied. Notably, over the 2 h incubation, isoflavones exhibited increased effect on the concentration of *Bifidobacterium*, *F. prausnitzii*, and Bacteriodetes; however, they decreased the concentration of Firmicutes. This might be significant in the *in vivo* fermentation process that occurs in the human intestine. The temporary exposure of bacteria in a gut region (e.g., ileum) to isoflavones is short (2–3 h), and a certain gut region has a specific gut bacterial compositions because of the different oxygen tensions at different places in the GI tract.[309, 310] However, in others *in vitro* experiments, a longer exposure time (12–48 h)[311] with such xenobiotics (e.g., isoflavones); this might result in an exaggerated effect of test chemicals on some specific groups of gut bacteria.

Chemically, flavonoids are further divided into subclasses (e.g., flavones, isoflavones, and flavanols), and they are regarded as prebiotics present in the diet.[312] Flavanols (e.g., quercetin) have been shown anti-proliferative activity on Gram-negative bacteria (most pathogenic bacteria are Gram-negative); this is likely due to the ring fragmentation of flavanols during their intestinal metabolism to produce the corresponding phenolic acids.[170, 187] For example, quercetin can be broken down into hydrocattetic acid and 4-hydroxyphenylpropionic acid (Fig. 6.3).[170] However, with a different molecular skeleton (i.e.,

different relative positions between A ring and B ring, Table 1.2), isoflavones are rarely found to be fragmented into their corresponding phenolic acids.[229, 285]

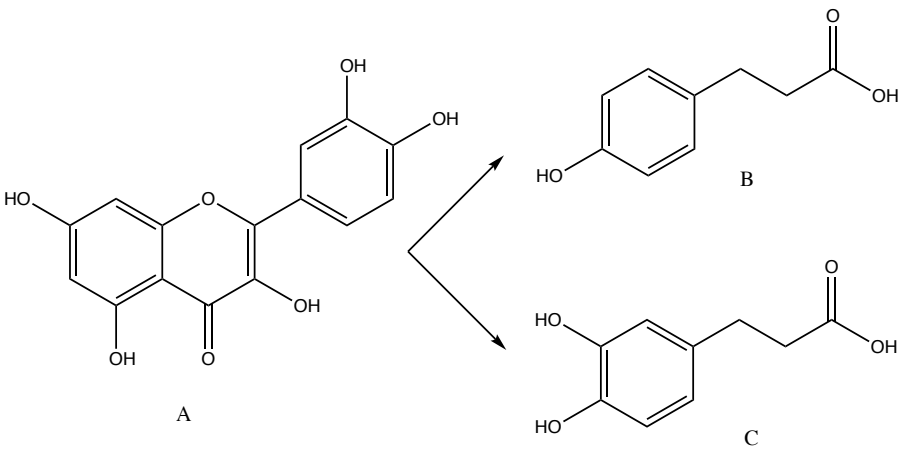


Figure 6.3: The metabolism of quercetin (A) by gut bacteria to form 4-hydroxyphenylpropionic acid (B) and hydrocattetic acid (C).

6.4.2 Transport study of isoflavones in Caco-2 cell monolayer system

There were seven isoflavones with different hydroxyl patterns studied in the Caco-2 cell monolayer model system. These isoflavones were rapidly transported and metabolised in the transportation experiment. The apparent transport rates (TRs) of the individual isoflavones are different and range from 13.59 to 25.2 pmol/min/cm^2 (Fig 6.5). The transport rate (TR) of GEN is fastest, followed by DAID (17.87 pmol/min/cm^2), 3′-OH-GEN (17.58 pmol/min/cm^2) and 8-OH-GEN (17.73 pmol/min/cm^2) which are similar. 6-OH-DAID (14.04 pmol/min/cm^2), 3′-OH-DAID (13.39 pmol/min/cm^2) and 8-OH-DAID (14.0 pmol/min/cm^2) (Fig. 6.5). In addition, the P_{app} of the studied isoflavones (Fig. 6.4) follows the same trend as their transport rates, ranging from 24.5 to 37.2 $\times 10^{-6}$ cm/s (Fig. 6.4). In summary, the transport across the Caco-2 monolayer of GEN and DAID are faster than their corresponding hydroxylated compounds, and the OH-GENs are faster than OH-DAIDs.

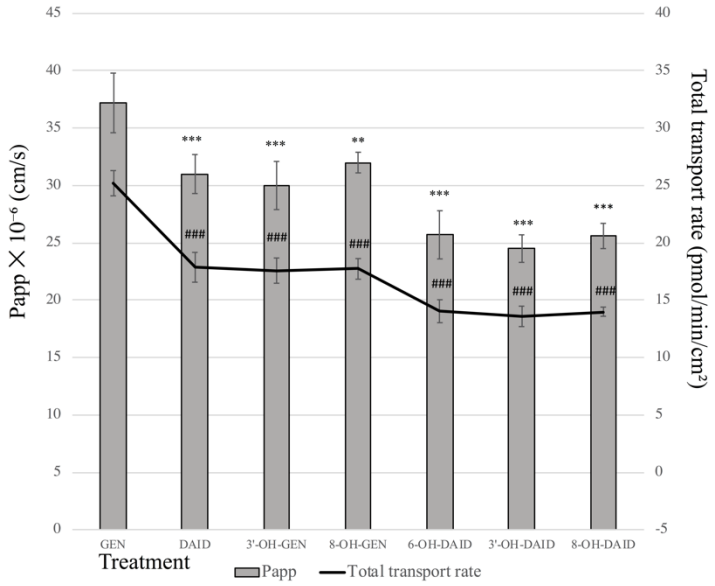


Figure 6.4: P_{app} and total TR of isoflavones (mean ± SD, n = 3). P_{app} and total TR were measured in Caco-2 cell monolayer system. *Significant difference in the P_{app} value (**: $p<0.03$; ***: $p<0.01$), # significant difference in the TR (###: $p<0.01$), both of which were comparisons of each compound with GEN calculated by one-way ANOVA.

Intestinal phase II metabolism (e.g., sulfation and glucuronidation) is important metabolic pathways of xenobiotics (e.g., flavonoids); the conjugation of xenobiotics has the potential to facilitate their excretion to the lumen of the intestine.[313] Figure 6.6 shows the transport rates of conjugates and aglycones of the studied isoflavones. All the aglycones of the studied isoflavones were absorbed by Caco-2 cells. GEN has the fastest TR (18.67 pmol/min/cm^2), followed by DAID (13.58 pmol/min/cm^2), and the slowest compound was 3′-OH-DAID (7.07 pmol/min/cm^2). Interestingly, there is no significant difference in the conjugate transport rates between individual studied isoflavones; this might be due to their structural features, namely—same molecular skeleton and common substitution arrangements (Fig. 1.12). This finding is also supported by previous research of regioselective sulfation and glucuronidation of phenolic,[313] this research shows structural basis for the conjugation of

isoflavones is the 7-hydroxy group is the major site for glucuronidation whereas the 4′-hydroxyl group is the only possible site for sulfation. Indeed, both the 7-hydroxyl and 4′-hydroxyl are common structural features of all isoflavones (Table 1.10).

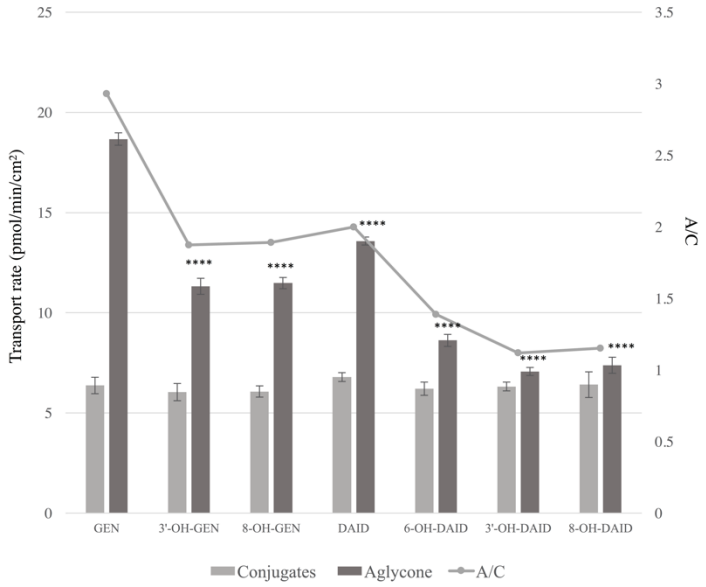


Figure 6.5: TR of conjugates and aglycone (mean ± SD, n = 3). *Significant difference in the TR of aglycone (****: p<0.001), which was the comparison of each compound with GEN calculated by one-way ANOVA.

In addition, the plotted line in Figure 6.5 shows the ratio between the absorbed aglycones and their corresponding conjugates in the Caco-2 cell monolayer system, which ranges from 1.11 to 2.9.; this follows the trend of the TR of the aglycones of the isoflavones studied. This finding suggests the structural features of isoflavones could influence the absorbed of their corresponding aglycones (functional forms), and, thus, an effect their bioactivity *in vivo*. Furthermore, the amount of absorbed hydroxylated isoflavones in the Caco-2 monolayer system was less than the amount of their corresponding parent compounds absorbed (Fig. 6.5); for example, the aglycone TR of 8-OH-GEN is 11.42 pmol/min/cm^2, while the aglycone TR of its parent compound—is 18.67 pmol/min/cm^2. This means the hydroxylated isoflavone metabolites may have less functional forms than their corresponding parent compounds,

which imply the hydroxylated biotransformation in food fermentation may results a reduction of bioavailability of isoflavones. This trend could be important in a cellular bioactivity or human health setting. This is exemplified by recent evidence suggested that interaction exists between estrogens (including endogenous estrogens and xenoestrogens) and gut health (e.g. colon carcinogenesis).[270, 314] Isoflavones in the diet are the main source of phytoestrogen exposure, which is shown to stimulate the proliferation of human colon cells at low exposure concentrations in this thesis (***Section 5.4.2, Chapter 5***), but to inhibit cellular proliferation at high exposure concentrations in previous research[271]. Importantly, isoflavones have a higher binding affinity with ERβ, which is the predominant ER form in the GI tract. Bringing all these facts together suggests the structural features of isoflavones might determine the absorption and metabolism of isoflavones, the absorbed aglycones of isoflavones might affect gut health *via* ERβ mediated bioactivity. This is exemplified by the ERβ-mediated proliferation of gut cell, which influences the integrity of the GI tract, and, thus, affect the functionality of gut.[315]

6.5 Conclusions

Soy isoflavones are phytoestrogens which can occupy and activate ERs due to their structural mimicry of E2. In this chapter, seven isoflavones including GEN, DAID, and their hydroxylated metabolite, have been studied.

Firstly, the *in vitro* fecal fermentation experiment showed that the studied isoflavones have different effects on populations of target groups of gut bacteria (i.e., Bacteroidetes and Firmicutes), one species of Firmicutes—*F. prausnitzii*, and a species of principal beneficial commensal bacteria—*Bifidobacterium* spp., and respectively). Briefly, over 2 h of the fermentation, isoflavones increased the concentration of *Bifidobacterium* spp, *F. prausnitzii*, and declined the F/B rapidly. The inulin control group continued increasing the concentration of *Bifidobacterium* spp, *F. prausnitzii*, and declined the F/B. Over the 2 h incubation, except 6-OH-DAID, all isoflavones have significant greater ($p < 0.03$) proliferative activity on the concentration of *Bifidobacterium* ranging from 6.87 $\log_{10}$ cfu/mL (8-OH-GEN) to 7.08 $\log_{10}$ cfu/mL (3′-OH-DAID) compared with the inulin control (6.24 $\log_{10}$ cfu/mL), but GEN and DAID do not show any significant difference compared with their corresponding hydroxylated metabolites (Fig. 6.1). In addition, all studied isoflavones show stronger inhibitory effects on F/B (ranging from 1.36 to 1.54) than inulin control (1.56) in 2 h (Table 6.1); 3′-OH-DAID and 3′-OH-GEN have the highest potency of reduction on F/B (Table 6.1). Interestingly, DAID and GEN do not show any significant differences in the effect on the concentration of *F. prausnitzii* compared with their corresponding hydroxylated metabolites. The exception of this finding is 8-OH-DAID, which exhibits significant greater effect on the concentration of *F. prausnitzii* compared with DAID. In addition, Only 6-OH-DAID (6.60 $\log_{10}$ cfu/mL, $p < 0.03$) shows significant greater effect on the concentration of *F. prausnitzii* compared with the inulin control (5.74 $\log_{10}$ cfu/mL) (Fig. 6.2).

The transport experiment shows that all studied isoflavones can be absorbed across the Caco-2 monolayer system and conjugated by phase II metabolism in the cells. The apparent

transport rates indicate that the transportation of GEN and DAID are faster than their corresponding hydroxylated compounds, respectively; the OH-GENs are transported faster than OH-DAIDs. Interestingly, there is no significant difference between these isoflavones in terms of their conjugate transport rates. This is likely due to the regioselective sulfation and glucuronidation on the characteristic structural features of isoflavones (i.e., 7-hydroxy and 4′-hydroxy). The Caco-2 cell line mimics the differentiating gut enterocytes and models the function for absorbing xenobiotics (e.g., isoflavones).[277] The amount of aglycones (transported by Caco-2 cell monolayer from AP to BL) of hydroxylated isoflavones are less than their corresponding parent compounds. This could be important in a cellular bioactivity or even human health setting. Food fermentation would convert GEN and DAID in their hydroxylated metabolites, and influence the gut cell transportation of aglycones which are the functional forms. Aglycones might interact with macromolecules in cells (e.g., ERs) thus having biological effects (e.g., estrogenic regulation), and thus; the amount of absorbed aglycone might determine the functionality of dietary isoflavones.

Chapter 7: Overall Discussion and Concluding Remarks

7 Chapter 7 – Overall Discussion and Concluding Remarks

7.1 Communication between the LBC of ERs and their AF-2 site is triggered by the docked ligands

ERs have functions beyond traditional ideas of estrogen activity.[20] Not only do they initiate and guide sexual development, they also have key roles in stimulating cell division; for example, early neurological development.[14, 316] All of this is achieved by a specialised region of the receptor—LBC, which interacts with estrogens to cause conformational changes in the receptor and in turn leads to receptor/ligand complex dimerization followed by occupancy of a DNA region (ERE) which controls gene expression of transactivation genes (Fig. 3.1).[19, 23] ***Chapter 3*** reports that, LBC and AF-2 are two connected binding sites in the LBD of ERs. A ligand docks at the LBC and induce conformational change, which rearrange the conformation of the LBD; this results in ripple effects on the structural components of AF-2 *via* its sharing loop or helices with the LBC (e.g., H11 or H12). The "triangular relationship" involving two binding sites (i.e., the LBC and AF-2) and the bound ligand[24] could explain the communication between the LBC and AF-2 which facilitates fine tuning of the estrogenic response.

7.2 Individual ligands docked at the LBC can cause different knock-on effects on AF-2

Chapter 3 indicates that individual ligands with different structural features (e.g., substituent bulkiness) can initiate various conformational changes in the LBC. Aligning X-ray crystallographic data of different complexes of ligand/ERα reveals that LBD has intimately interactive components (i.e., LBC and AF-2), the LBC is open to abuse by non-natural ligands of ERα that can also interact with AF-2. For example, GEN docked at the LBC and

slightly shifted the orientation of His 524 compared with the complex of E2/ERα (Fig. 3.6). In addition, the protein alignment study in ***Chapter 3*** shows docked ligands with bulky side substituents have much greater effects on the conformation of the LBC with a consequently greater communication effect to AF-2. A breast cancer drug—raloxifene is a good example as an antagonist ligand of ERα.[20] In the complex of ERα/Raloxifene, the H12 is pushed away by the ethylpyridine side group of Raloxifene (Fig. 3.13); this destroys the harbour (i.e., AF-2) for accommodating regulatory proteins. The alignment of the complex of ERα/E2 (PDB entry code: 1ERE) and ERα/ Raloxifene (PDB entry code: 1ERR) might differentiate agonist and antagonist in terms of ERα's conformation. However, the comparison between two complexes of ERα with different agonist compounds (e.g., E2 (PDB entry code: 1ERE) and OBHS (PDB entry code: 5U2D) indicates significant insight into the intimate interplay of the receptor: the knock-on effects of these two ligands on AF-2 are different and resulting in different amino acid residue arrangements in the AF-2 (e.g., the orientation and distribution of Glu-380) (Fig. 3.11). This means the binding environment of AF-2 for regulatory proteins depends on LBC docked ligands. Interestingly, a closer look at the spatial arrangement of amino acid residues at AF-2 when GEN or ETP is docked shows subtle, but important differences; for example, Glu-542 on H12 is re-orientated when ETP is *in situ* compared to GEN (Fig. 3.13). The different orientations of Glu-542 influence the distance or angle between the donor (i.e., Glu-542) and the acceptor (Leu-2004) on a H-bond thus determine the presence of H-bonds between the AF-2 and the GRIP-peptide (a fragment of a regulatory protein) (Fig. 3.14). Occupancy of AF-2 by a regulatory protein is important in modulating ERα's biological activity.[16] Regulatory proteins are histone acetyltransferaes-acetylation rearranging the chromatin structure, exposing the target genes to the transcriptional machinery.[16] Recruitment of regulatory proteins influences this process, leading to gene transcriptional changes, which might underlie different estrogenicities.[16] Furthermore, regulatory protein recruitment controls ERα degradation *via* phosphorylation of Tyr537.[179] This phosphorylation signals initiation of the proteasome ubiquitination pathway, leading to polyubiquitinated ERα which is transported to the proteasome for degradation.[180] The regulatory protein recruitment influences the ubiquitination process, leading to differential downstream bioactivity effects; for example, cell proliferation and cell migration.[179] In

summary, the conformational differences resulting from ligands' (e.g., GEN or ETP) docking at the LBC likely alter ERα's biological response—this might explain the molecular interplay between LBC and AF-2 determining ERs' activities. For example, previous research indicates that ERα expressed in the hypothalamus mediates antiobesity effects of estrogens in females, and the interaction between ERα and a coactivator protein (steroid receptor coactivator-1) has been shown to mediate the antiobesity effects of E2-ERα signals.[317] Bringing the thoughts in this section suggests that different ligands (e.g., GEN) when docked at the LBC lead to a conformational change which result in different amino acid residue arrangements in the AF-2, thus differentiating the interaction of coactivators (e.g., steroid receptor coactivator-1) with ERα; in turn, this influences the functionality of ERα *per se* (e.g., antiobesity).

7.3 The interactions of studied isoflavones with ERα and ERβ are different

The comparison of the LBD of ERα and ERβ can be illustrated by the alignment of ERα/GEN crystal (PDB entry code: 1ERE) and ERβ/ GEN crystal (PDB entry code: 1ERR) (Fig. 1.2); ***Chapter 1*** indicates the two isoforms of ER have similar helices architecture (Fig. 1.2), which means the interrelationships between each helix in ERα and ERβ are similar. However, the overall homology between ERα and ERβ is surprisely less than 55%.[52] Intriguingly, the amino acid residues comprising the LBC of ERs are highly homologeneous.[52] Figures 1.7 & 1.8 show that ERα and ERβ provide a similar compact hydrophobic pocket for hosting ligands, but have subtle differences in amino acid residue orientations; for example, the relative position of histidine (His 524 in ERα; His 475 in ERβ) and threonine (Thr 347 in ERα; Thr 299 in ERβ) in the LBC. This suggests a ligand may facilitate different noncovalent interactions docked in ERα and ERβ, resulting in different binding energies. The *in silico* studies of isoflavones in ***Chapters 4*** & ***5*** predict that the ligand candidates (i.e., isoflavones) can dock in both ERα and ERβ, but with different theoretical

binding energies represented by their DockingScores. Interestingly, an isoflavone (e.g., GEN) can form the same number of H-bonds with ERα and ERβ but having different H-bond values (i.e., H-bond energy) (Table 7.1). This is likely due to the different orientations of amino acid residues in the LBC between ERα and ERβ, which determines the angle and distance between the donor and acceptor atoms in an H-bond, and, thus results in different H-bond values.[217] Results of ***Chapters 4 & 5*** indicate that, since H-bond values may influence the binding affinity and corresponding receptor-driven activity, a ligand interact with different receptors (i.e., in this thesis, ERα and ERβ) having different H-bond values, thus may result in different estrogenicities. In addition, the subtle differences of arrangements of amino acid residue affect the hydrophobic interactions between the docked isoflavones with the LBC and result in different HER values (Table 7.1). This shows a trend that isoflavone's HER docked with ERβ are greater than docked with ERα, but their corresponding DockingScores do not follow the same trend. For example, DAID has a higher DockingScore with ERβ (−11.03 kcal/mol) than with ERα (−10.49 kcal/mol); but 6-OH-DAID has a lower DockingScore with ERβ (−11.88 kcal/mol) than with ERα (−11.99 kcal/mol) (Table 7.1). This finding indicates that isoflavones might facilitate greater hydrophobic interaction with ERβ compared with ERα. Importantly, the predicted binding energies of isoflavones with ERα and ERβ suggest isoflavones have binding selectivity with different ERs; this indicates isoflavones' bioactivities may differ in different tissues because of the varying ER tissue distributions (e.g., ERα is predominantly in breast, ERβ is predominantly in the GI tract), and thus, the functionality of isoflavone-containing foods may be tissues-selective in humans because of their ER isoform preferences in tissues.

From a food functionality perspective, it is important to integrate both dose-related biological effects of isoflavones and the difference in tissue distributions of ERs. The complex interplay between these factors will determine specific tissue-isoflavone related effects. For example, GEN has breast cancer inhibitory effects at high doses and promotes the proliferation of MCF-7 cell in culture at low doses.[93] The balance between dietary phytoestrogen and endogenous estrogen levels should also be considered; for example, isoflavone supplements

are sometimes used to alleviate symptoms of menopause[318] because they enhance total plasma estrogenicity, thus ameliorating the biochemical and physiological effects of declining natural estrogens as the menopause progresses. On the other hand, women with high circulating estrogen levels (e.g., child-bearing age women) would be unlikely to benefit from isoflavone dietary supplementation because they would increase the total circulating estrogenicity. A similar argument applies to the implications of dietary phytoestrogen to breast cancer risk where high isoflavone (e.g., GEN) doses appear to prevent breast cancer cell proliferation, whereas low doses promote proliferation.[122] Importantly, GEN can interfere with the action of ERα-based treatments for breast cancer (e.g., tamoxifen) because they might compete with tamoxifen for occupancy of the LBC; [319] this suggests isoflavone metabolites (e.g., 6-OH-DAID), which have been shown greater predicted binding affinity with ERα than GEN in ***Chapter 4***, may have a greater impact on ERα-based treatments for breast cancer.[319] In addition, the greater interaction of some isoflavones (e.g., DAID, GEN) with ERβ should draw attention to their ERβ-driven activity in predominately ERβ expressing tissue (e.g., GI tract).[270, 320]

Table 7.1: Comparison of docking studies of isoflavones bound to ERα and ERβ.

Compounds	Docked at ERα			Docked at ERβ		
	DockingScore (kcal/mol)	H-bond value (kcal/mol)	HER (kcal/mol)	DockingScore (kcal/mol)	H-bond kcal/mol	HER kcal/mol
DAID	−10.49	−1.26	−4.90	−11.03	−1.21	−5.68
GEN	−10.79	−1.7	−4.8	−11.45	−1.67	−5.52
6-OH-DAID	−11.99	−2.42	−4.99	−11.88	−2.18	−5.57
8-OH-DAID	−11.62	−2.27	−4.97	−11.56	−1.72	−5.64
3′-OH-DAID	−11.5	−2.13	−5.01	−11.85	−1.92	−5.64
8-OH-GEN	−11.68	−2.5	−4.6	−11.72	−2.18	−5.47
3′-OH-GEN	−11.8	−2.59	−4.71	−11.64	−1.66	−5.38

7.4 The structures of isoflavones determine their ER-driven activities

Chapters 4 & 5 indicate that the induvial structural features of ligands determine their theoretical binding energy and binding affinity and might affect their ER-driven activities. In

this thesis, the *in silico* study of isoflavones with different hydroxyl arrangements indicates the structure binding energy/affinity relationships of ligands with ERα and ERβ; the hydroxylated metabolites (e.g., 6-OH-DAID and 3′-OH-GEN) of DAID and GEN have higher binding energies and greater affinities with either ERα or ERβ, thereby resulting in greater bioactivities in both the MELN assay and Caco-2 cell proliferation studies compared with the corresponding parent compound (i.e., DAID or GEN) (Tables 4.5 & 5.3). Bringing these results together, the increased bioactivity of these hydroxylated metabolites is likely due to their additional hydroxyls, which might form extra H-bonds with amino acid residues at the LBC (Figs 4.3 & 5.3). The exception to this trend is 8-OH-GEN; this compound is predicted to have higher binding energy and greater binding affinity than the parent compound GEN (Tables 4.6 & 5.1); however the additional hydroxyl groups on 8-OH-GEN decreases the hydrophobicity of the ligand resulting in lower HER compared with the less hydroxyl parent compound (Tables 4.3 & 5.1), and do not contribute any polar interaction (i.e., H-bond interaction) at the LBC (Fig. 7.1); this might result in weaker ER-driven activities (Tables 4.5 & 5.6). So, contrary to expectations the extra hydroxyl group resulting the molecular hydrophobicity in a key region of the ligand results in less favourable interaction with the LBC and likely lowers bioactivity.

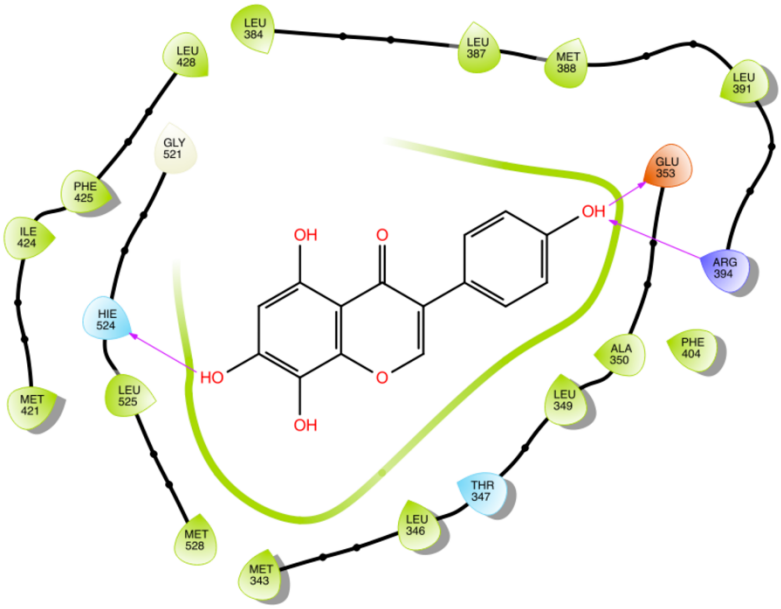


Figure 7.1: Interaction between 8-OH-GEN and the LBC of ERα from *in silico* study showing the free hydroxyls decrease the molecular hydrophobicity of the ligand and are not favour for the hydrophobic region of the LBC. Purple arrows represent H-bonds; the direction of arrow denotes electron donor to electron acceptor in the H-bond. Different coloured residues represent amino acid properties: blue = positive charge; red = negative, cyan = polar, green = nonpolar. (NB: Schrödinger uses a non-standard amino acid abbreviation system: HIE = Histidine).

Caco-2 cells comprise a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma cells and with phase II metabolising capacity.[321, 322] The intestinal Phase II metabolism is important in isoflavone deactivation—isoflavones are glucuronic acid conjugates *via* their hydroxyl group.[277,269, 323] In this thesis, ***Chapter 6*** also proves that Phase II conjugation of isoflavones occurred in the Caco-2 monolayer transportation experiments in this thesis. The pre-treatment with GA, an inhibitor of UGDH, enhanced GEN or 8-OH-GEN mediated Caco-2 cell proliferation. This suggests that GA treatment interferes with the phase II conjugation of isoflavones resulting in an increase of amount of functional aglycones in the Caco-2 cells. Interestingly, ***Chapter 5*** indicates that a "food cocktail" effect involving fruits containing GA and soybeans containing isoflavones may lead a complex food functionality. GA from fruit or tea might inhibit the activity UGDH and decrease the biosynthesis of UDP

glucuronic acid, which would provide glucuronic acid for the conjugation of soy isoflavones (Fig. 7.2).[269] This would interfere with the conversion of isoflavones to non-bioactive conjugates and result in an increase in the aglycone concentration leading to this modulation ER-driven bioactivities (e.g., in gut) (Fig. 7.2). The fact that ERβ is the predominant ER isoform in gut draws more attention to the potential importance of ERβ in gut health.[64, 259, 263] On the positive side, ERβ mediates gut cell proliferation, and the cell proliferation plays an important role in the maintenance of the integrity of GI tract, which has linked to the immunity, digestion of GI tract.[324, 325] Collectively results from ***Chapter 5*** indicate isoflavone intake may benefit gut health *via* its proliferative effect on gut cell, and in combination with GA may increase the positive effect of isoflavone on gut health. On the negative side, for example, gut transit is associated with ERβ, the delayed gut transit time during pregnancy has been associated with high circulating estrogenicity because of the high level of E2.[266] During this period, isoflavone intake may worsen gut transit; the food combination including isoflavone and GA may cause additive ERβ-mediated effect, thus amplify the situation.

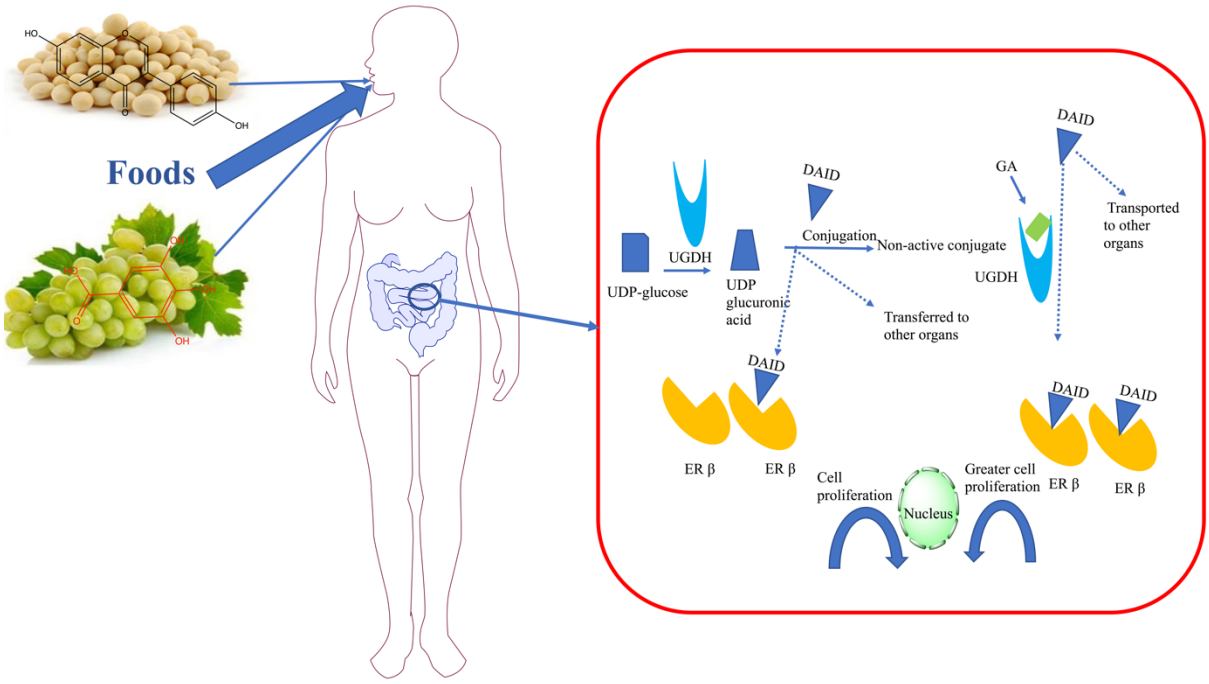


Figure 7.2: Schematic to show the complex dietary cocktail effects involving DAID and GA from different foods, and leading different proliferative effects of isoflavones on gut cells.

This finding is also important and might be helpful in reducing foo-related risks; for example, a breast cancer patient would want to avoid the additive ER-driven bioactivities caused by the complex dietary intake of GA and isoflavones, because isoflavones may interfere with the tamoxifen treatment, and therefore, it might result in ERα positive breast cancer cell proliferation; On the other hand, the additive ER-driven bioactivities may be beneficial for menopausal females to alleviate their menopausal symptoms caused by the natural reduction of estrogens.

7.5 The interrelationships between food isoflavones, gut bacteria, and gut cells, and potential health consequences

7.5.1 The interaction between gut bacteria and isoflavones

Isoflavones can be metabolised by gut bacteria in the human intestine.[282, 285, 326] The glycoside form of isoflavones (e.g., daidzin), which normally exist in raw soy, are hydrolysed to release the functional form (i.e., aglycone) of the isoflavone (e.g., DAID).[285] In addition, the gut bacterial metabolism produce different estrogenic metabolites compared with the corresponding parent compound; DAID is metabolised to the more estrogenic *S*-equol (Fig. 3.3);[190] and GEN is converted to the less estrogenic dihydrogenistein (Fig. 3.10).[223] ***Chapter 6*** reports a *in vitro* gut fermentation study of isoflavones and showing the effects of isoflavones on the concentrations of two phyla, Bacteroidetes and Firmicutes, the concentrations of a specific species of Firmicutes—*F. prausnitzii*, and *Bifidobacterium* spp. This indicates the bi-directional interaction between gut bacteria and isoflavones. This means that gut bacteria can metabolise isoflavones so changing their potential bioactivity while also changing the bacterial population in response to the isoflavone. So, in a dynamic context, the bacteria metabolise isoflavones which change their effect on the bacterial carrying out the metabolism.

7.5.2 The interaction between isoflavones and gut cell

Results in ***Chapter 6*** show that all isoflavones studied (i.e., aglycones) were taken up by the Caco-2 cell monolayer system, since this mimics the human intestinal mucosa, this likely that these isoflavones are also taken up by human gut cells *in vivo* (***Chapter 6***). Following uptake, the aglycones (functional form) can be conjugated by phase II metabolism (e.g., sulfation and glucuronidation). It has been suggested that, in the gut cell, the aglycones might interact with macromolecules (e.g., ERs) or be transported to other tissues;[277] this indicates that the phase

II metabolism of isoflavones determines the functionality of isoflavones and influences macromolecules-driven activities (e.g., *via* ER interactions). As discussed before, ERβ is the predominant ER isoform in the GI tract, and ***Chapter 6*** shows isoflavones have been shown to be absorbed by the Caco-2 monolayer system;[64] this suggests the potential interactions between soy isoflavones and ERβ could be significant in a cellular context. The Caco-2 proliferation experiments in ***Chapter 5*** showed isoflavones have a stimulatory activity on the proliferation of the cells. This ERβ-driven activity of isoflavones in Caco-2 cell might have more significance in a human health level. For example, the higher incidence of Crohn's disease in women[327, 328] and more frequent acute episodes of disease activity in women[329] points to a possible E2 link. The dominant role of ERβ in small bowel mucosal cells, particularly in the terminal ileum, could be significant given that exposure to E2 would occur *via* its excretion in bile. In addition, in ***Chapters 4 & 5*** , selected isoflavones have been shown to be estrogen mimics in both *in silico* and in *in vitro* studies, and shown to increase the concentration of *F. prausnitzii*, which has been associated with Crohn's disease. This suggests the combination of ERβ-driven activity and the effect on bacteria of isoflavones may be important in the aetiology of Crohn's disease, and this dietary manipulation might play a role in the treatment of Crohn's disease. Thus, isoflavone may become a potential treatment or specific functional food for Crohn's disease.

7.5.3 Gut health might determine the expression of ERβ

The potential role of ERβ in the GI tract is attracting considerable attention, since the presence ERβ and its mRNA has been identified in rat, monkey and human colon tissues;[51, 183, 270, 330] but the influence of gut health on the expression of ERβ is rarely studied. A limited example is the colonic tumorigenesis, which results in decreased ERβ mRNA steady-state levels compared with normal colon mucosa cells;[51] this might influence the expression of ERβ, and thus affect ERβ bioactivity in the gut. This might indicate that gut disease could affect ERβ expression and affect the impact of dietary isoflavones on gut cell proliferation.

7.5.4 Isoflavones might interfere with the metabolism of endogenous estrogens and affect health

Estrogens undergo hepatic recirculation (Fig. 1.6).The aglycone forms of endogenous estrogens (e.g., E2) could undergo first-pass hepatic metabolism.[48] Hepatically conjugated estrogens excreted in the bile can be deconjugated by bacterial (e.g., Bacteroidetes and Firmicutes) β-glucuronidase activity in the gut, leading to the reabsorption into the circulation.[48] Interestingly, in ***Chapter 6*** , the *in vitro* gut fermentation experiment showed that isoflavones influence the concentration of Bacteroidetes and Firmicutes in the mixed culture and suggesting that the dietary isoflavones might influence the reabsorption of endogenous estrogens. This suggests that the isoflavones influence the total circulating estrogenicity in two ways. Firstly, isoflavones are estrogen mimics, a part of aglycone of isoflavones would be taken up by gut cell and circulated in bloodstream. Secondly, isoflavone may affect the estrogen reabsorption *via* modulating gut bacterial populations. This is significant as isoflavones have been shown to alleviate the symptoms caused by the loss of E2 (e.g., menopausal symptoms).

7.6 Future work

The complexity of gut microbial ecology, inter-individual host variability in both bacterial species and their distribution, and difficulties replicating human systems in animal models means that significant effort is needed to address and understand these issues. This could be achieved by:

- Studying microbial ecology in human normal gut sampled during cancer excision surgery using rRNA Gene Sequence technology.
- Maintaining the human gut samples in culture to allow isoflavone metabolism/uptake experiments to be conducted.
- Studying the effect of varying gut bacteria on isoflavone metabolism.

- Studying the effects of isoflavones in culture media on microbial replication and biochemistry.
- Studying the effects of isoflavone microbial metabolites on human cell systems (e.g., Caco-2).
- Extrapolating the findings to human gut-based diseases (e.g., Crohn's disease, gastrointestinal tract cancer).

At the other end of the spectrum, it is necessary to understand better the interaction of isoflavones and their metabolites with ERs to enable prediction of biological activity. This could be achieved using dynamic modelling systems such as DESMOND which facilitate molecular plasticity which overcomes the constraints of rigid Schrödinger docking.

7.7 A integrated approach to gut health, the microbiome and functional food components

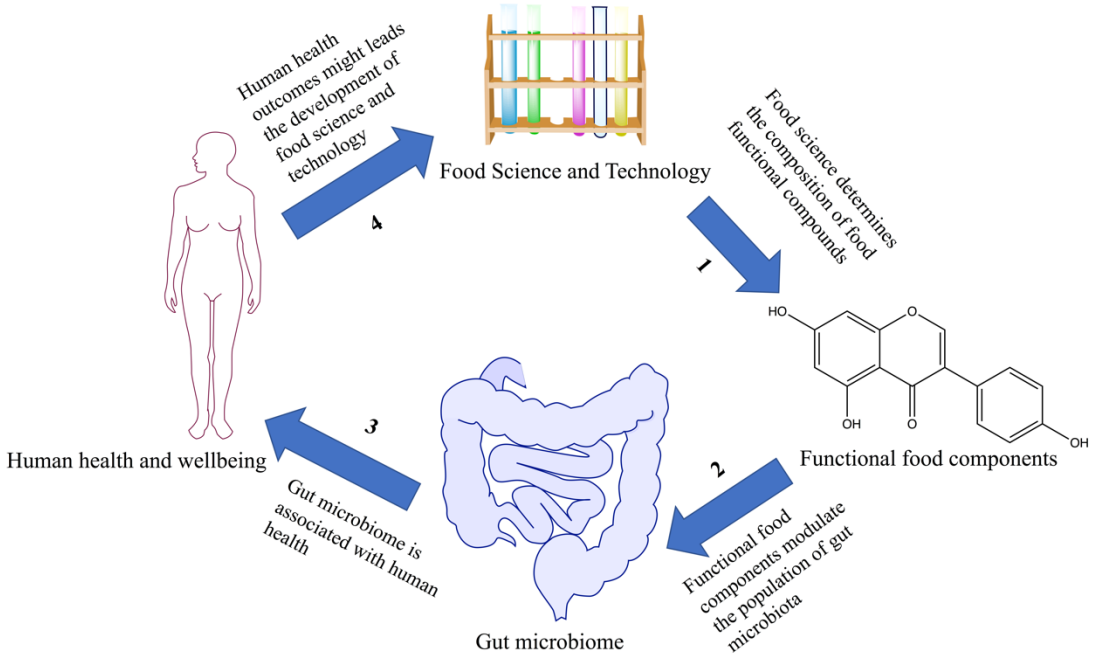


Figure 7.3: The interrelationships between food science and technology, functional food components, gut microbiome, human health and wellbeing. Morden food science and technology enables manipulation of food to modify their components and functionality (1). This functionality might modulate the population of gut microbiota (2) and influence human health and wellbeing (3). The human health outcomes might lead the development of functional foods by food science and technologists (4).

Food science technology influences the compositions of food's functional compounds.[331] A good example is the changes in the composition of isoflavones during tofu, a soy-based food's production.[11] The production of tofu includes soaking, grinding, filtering, boiling, and coagulating. The different ratios between water and soybeans during grinding leads to variation in tofu isoflavone compositions;[152] and heat treatment in the boiling process has been reported to convert some malonyl isoflavones into their corresponding acetyl forms.[332]

In addition, calcium sulfate has been reported as the best coagulant for tofu manufacture because in maintains the natural isoflavone compositions.[154] What's more, during food fermentation, DAID and GEN are converted into to two types of biotransformation products: OH-DAID and OH-GEN;[157, 251, 252] this biotransformation is a regiospecific hydroxylation which depends on the specific of CYP450 in microorganism used in food fermentation process (Table 7.2).[253, 254] This suggests that, in food fermentation, using specific microorganism could yield different isoflavone hydroxylated metabolites with different bioactivities of, and thus, modulate food functionality. This varying the bacteria species used in fermentation-based food manufacturing processor could affect the functionality of the resulting food products. For example, to enhance the alleviation of isoflavones on menopausal symptoms, specific microorganism can be used in food fermentation to produce more isoflavone metabolites with greater estrogenicity.

Table 7.2: Hydroxylated metabolites of DAID and GEN, food sources, and microorganism used in food manufacture.[253, 254]

Fermented soy food	Microorganism used	Metabolites of DAID and GEN
Tempeh	*Rhizopus* and other bacteria	6-OH-DAID, 8-OH-DAID, 3′-OH-GEN
Soybean koji	*Aspergillus saitoi*	8-OH-DAID, 8-OH-GEN, 6-OH-DAID
Miso	*Aspergillus oryzae*	8-OH-DAID, 8-OH-GEN. 6-OH-DAID, 3′-OH-DAID
Douchi	*Aspergillus oryzae*	6-OH-DAID, 8-OH-DAID, 3′-OH-DAID, 8-OH-GEN
Doenjang	Diverse fungi and bacteria	6-OH-DAID, 8-OH-DAID, 3′-OH-DAID

The gut microbiome comprises tens of trillions of bacterial cells.[189] A major function of these bacteria is to digest food ingredients (including food functional compounds) so that the products of the intestinal biotransformation can be utilized in beneficial ways to support myriad aspects of human biology.[170] Some non-digestible food functional compounds are described as “prebiotics” which beneficially affect the host by selectively “stimulating” the growth and /or activity of one or a limited number of bacteria in the GI tract and thus might

improve host health.[189] Some well-known examples of prebiotics include the dietary polysaccharides which can produce SCFAs by bacterial metabolism;[333] the plant flavonoids which have been reported to have pharmacological properties including anti-proliferative effect on Gram-negative bacteria (most pathogenic bacteria are Gram-negative).[334] A good example of prebiotics in ***Chapter 6*** is that isoflavones can increase the amount of *F. prausnitzii*, these bacteria have been associated with the pathophysiology of Crohn's disease.[306] Interestingly, GEN and DAID from raw soy or soy milk have a greater effect on the growth of *F. prausnitzii* compared with their hydroxylated metabolites from fermented soy foods (e.g., soy sauce, miso). This suggests fermented vs non-fermented food manufacturing processes might alter the compositions of food functional compounds and varying their effects on the populations of specific gut bacteria, which, in turn, might influence health.[335, 336] A great deal of evidence indicates the connections between gut bacteria and human health. For example, gut bacterial communities have been suggested to contribute to the development of insulin resistance,[53] and the dynamic of gut bacteria has been associated with immune function due to their effect of intestinal permeability.[297, 298] In addition, gut-brain axis seems to be bidirectional-the brain acts on GI and immune functions that help to modulate the gut's microbial makeup, and gut bacteria make neuroactive compounds, including neurotransmitters and metabolites that also act on the brain.[337] The ongoing exploration of gut bacteria promises to bring the "gut-brain axis" into a clearer focus.[338, 339] Research is clearly pointing that bacteria being a key component of health, it might be the right time to include the dynamic of bacterial populations and the effects of the dietary component (e.g., prebiotics) on gut bacteria as part of health and wellbeing strategies.[340, 341]

In future, it might be possible to develop food functionality for a specific purpose with foods "designed" to contain specific functional components for a desired positive health outcome. In a functional food context, the ingredient list of food functional compounds would reflect the fact that the food contains components designed to deliberately manipulate gut bacteria in a selective manner so as to benefit one or more facets of *in vivo* biology, with resulting

improvements in health status. The development of synergism between physiological and technical should be given more attention. Food technology will be able to manipulate and exploit the synergistic effect of tailored food components, which enhance functionality of a or a few targeted food components, on physiological functionality leading to positive health effects; advanced analytical techniques should be employed to tract and quantify food functional components before processing the manufacture of foods. For example, the circulating estrogen levels differ between gender and age. It is feasible to quantify the concentration of isoflavones in soy-based foods, this would cater to different gender-basis or age-basis requirements of total circulating estrogen levels *via* modulating the populations of gut bacteria. This leads to the modification of food process aiming to manipulate the compositions of food functional components. In short, these designer functional foods would be fashioned based on considerations of the food functional components' characteristics, availability and affordability, how the ingredients might be processed in ways that do not deleteriously affect the integrity and/or bioactivity of key nutrients and whether the manufactured food products will have acceptable organoleptic properties.

Getting back to isoflavones in food and their role in functionality and health. Isoflavone can modulate gut bacterial populations, this may influence reabsorption of estrogens. During the interaction between isoflavone and gut bacteria, the parent isoflavone might be converted to different and possible more estrogenic metabolites, these metabolites may have different ERβ-driven effects on the proliferation biochemistry of gut cell, this may in turn influence gut health. Then the gut health status might change the expression of ERβ. This is an incredibly complex cyclical interplay that moderates biological activity—this is a Pandora's Box of complexity with potentially profound health implications.

As Hippocrates (c. 460 BC– c. 370 BC) famously said: "let food be thy medicine and medicine be thy food.

食物是最好的药物

Reference

1. Betoret, E., Betoret, N., Rocculi, P., and Dalla Rosa, M. (2015) Strategies to improve food functionality: Structure–property relationships on high pressures homogenization, vacuum impregnation and drying technologies, *Trends in Food Science & Technology 46*, 1-12.

2. Siro, I., Kapolna, E., Kápolna, B., and Lugasi, A. (2008) Functional food. Product development, marketing and consumer acceptance—A review, *Appetite 51*, 456-467.

3. Cai, Y., Luo, Q., Sun, M., and Corke, H. (2004) Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer, *Life Sciences 74*, 2157-2184.

4. Lee, J., Jeung, J., Oh, M., Lee, B., and Choi, D. (2009) Curcumin Attenuates Airway Hyperresponsiveness and Inflammation in Murine Asthma Model, *Journal of Allergy and Clinical Immunology 123*, S56.

5. Mares-Perlman, J. A., Millen, A. E., Ficek, T. L., and Hankinson, S. E. (2002) The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. Overview, *The Journal of Nutrition 132*, 518S-524S.

6. Moeller, S. M., Jacques, P. F., and Blumberg, J. B. (2000) The potential role of dietary xanthophylls in cataract and age-related macular degeneration, *Journal of the American College of Nutrition 19*, 522S-527S.

7. Ksouri, R., Falleh, H., Megdiche, W., Trabelsi, N., Mhamdi, B., Chaieb, K., Bakrouf, A., Magné, C., and Abdelly, C. (2009) Antioxidant and antimicrobial activities of the edible medicinal halophyte Tamarix gallica L. and related polyphenolic constituents, *Food and Chemical Toxicology 47*, 2083-2091.

8. Adlercreutz, H. (1995) Phytoestrogens: epidemiology and a possible role in cancer protection, *Environmental Health Perspectives 103*, 103.

9. Velez, L. M., Abruzzese, G. A., and Motta, A. B. (2013) The biology of the peroxisome proliferator-activated receptor system in the female reproductive tract, *Current Pharmaceutical Design 19*, 4641-4646.

10. Zhang, Z. B., and Yang, Q. T. (2006) The testosterone mimetic properties of icariin, *Asian Journal of Andrology 8*, 601-605.

11. Jackson, C.-J., Dini, J., Lavandier, C., Rupasinghe, H., Faulkner, H., Poysa, V., Buzzell, D., and DeGrandis, S. (2002) Effects of processing on the content and composition of isoflavones during manufacturing of soy beverage and tofu, *Process Biochemistry 37*, 1117-1123.

12. Harris, D., Besselink, E., Henning, S., Go, V., and Heber, D. (2005) Phytoestrogens induce differential estrogen receptor alpha-or beta-mediated responses in transfected breast cancer cells, *Experimental Biology and Medicine 230*, 558-568.

13. Setchell, K. (1998) Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones, *The American Journal of Clinical Nutrition 68*, 1333S-1346S.

14. Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., and Warner, M. (2007) Estrogen receptors: how do they signal and what are their targets, *Physiological Reviews 87*, 905-931.

15. Kuhl, H. (2005) Pharmacology of estrogens and progestogens: influence of different routes of administration, *Climacteric 8*, 3-63.

16. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response, *Genes & Development 20*, 1405-1428.

17. Akingbemi, B. T., Sottas, C. M., Koulova, A. I., Klinefelter, G. R., and Hardy, M. P. (2004) Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells, *Endocrinology 145*, 592-603.

18. Delfosse, V., Grimaldi, M., Cavaillès, V., Balaguer, P., and Bourguet, W. (2014) Structural and functional profiling of environmental ligands for estrogen receptors, *Environmental Health Perspectives 122*, 1306.

19. Klinge, C. M. (2001) Estrogen receptor interaction with estrogen response elements, *Nucleic Acids Research 29*, 2905-2919.

20. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Öhman, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor, *Nature 389*, 753.

21. Murphy, L. C., Seekallu, S. V., and Watson, P. H. (2011) Clinical significance of estrogen receptor phosphorylation, *Endocrine-related cancer 18*, R1-R14.

22. Shaw, I. C. (2018) *Food safety: The science of Keeping Food Safe*, John Wiley & Sons.

23. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen, *Cell 95*, 927-937.

24. Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2002) Defining the" S" in SERMs, *Science 295*, 2380-2381.

25. Schulster, M., Bernie, A. M., and Ramasamy, R. (2016) The role of estradiol in male reproductive function, *Asian Journal of Andrology 18*, 435.

26. Simpson, E. (2003) Sources of estrogen and their importance, *The Journal of Steroid Biochemistry and Molecular Biology 86*, 225-230.

27. Wilson, J. D., George, F. W., and Griffin, J. E. (1981) The hormonal control of sexual development, *Science*, 1278-1284.

28. Schulster, M., Bernie, A. M., and Ramasamy, R. (2016) The role of estradiol in male reproductive function, *Asian Journal of Andrology 18*, 435-440.

29. Siiteri, P. K., and Macdonald, P. C. (1966) Placental Estrogen Biosynthesis During Human Pregnancy1, *The Journal of Clinical Endocrinology & Metabolism 26*, 751-761.

30. Saunders, P., Fisher, J., Sharpe, R., and Millar, M. (1998) Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis, *Journal of Endocrinology 156*, R13-R17.

31. Roselli, C. E., Abdelgadir, S. E., and Resko, J. A. (1997) Regulation of aromatase gene expression in the adult rat brain, *Brain Research Bulletin 44*, 351-357.

32. Dorrington, J., Fritz, I., and Armstrong, D. (1978) Control of testicular estrogen synthesis, *Biology of Reproduction 18*, 55-64.

33. Toniolo, P. G., Levitz, M., Zeleniuch-Jacquotte, A., Banerjee, S., Koenig, K. L., Shore, R. E., Strax, P., and Pasternack, B. S. (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women, *JNCI: Journal of the National Cancer Institute 87*, 190-197.

34. Wu, T., Mendola, P., and Buck, G. M. (2002) Ethnic differences in the presence of secondary sex characteristics and menarche among US girls: the Third National Health and Nutrition Examination Survey, 1988–1994, *Pediatrics 110*, 752-757.

35. Lacy, D., and Pettitt, A. J. (1970) Sites of hormone production in the mammalian testis and their significance in the control of male fertility, *British Medical Bulletin 26*, 87-91.

36. Kligman, I., and Rosenwaks, Z. (2001) Differentiating clinical profiles: predicting good responders, poor responders, and hyperresponders, *Fertility and Sterility 76*, 1185-1190.

37. L'Hermite, M. (2013) HRT optimization, using transdermal estradiol plus micronized progesterone, a safer HRT, *Climacteric 16*, 44-53.

38. Overpeck, J. G., Colson, S. H., Hohmann, J. R., Applestine, M. S., and Reilly, J. F. (1978) Concentrations of circulating steroids in normal prepubertal and adult male and female humans, chimpanzees, rhesus monkeys, rats, mice, and hamsters: a literature survey, *Journal of Toxicology and Environmental Health, Part A Current Issues 4*, 785-803.

39. Becker, J. B., Berkley, K. J., Geary, N., Hampson, E., Herman, J. P., and Young, E. (2007) *Sex differences in the brain: from genes to behavior*, Oxford University Press.

40. Treloar, A. E., Boynton, R. E., Behn, B. G., and Brown, B. W. (1967) Variation of the human menstrual cycle through reproductive life, *International Journal of Fertility 12*, 77-126.

41. Marrian, G. F. (1930) The chemistry of oestrin: The chemical nature of crystalline preparations, *Biochem J 24*, 1021-1030.

42. Diczfalusy, E., and Lindkvist, P. (1956) Isolation and estimation of “free” oestrogens in human placentae, *Acta Endocrinologica 22*, 203-223.

43. Ozen, M. (2004) Hormones, Genes, and Cancer, *American Journal of Human Genetics 74*, 192-193.

44. Merrill, R. C. (1958) Estriol: A Review, *Physiological Reviews 38*, 463-480.

45. SIITERI, P. K., and MacDonald, P. C. (1966) Placental estrogen biosynthesis during human pregnancy, Oxford University Press.

46. Freeman, E. R., Bloom, D. A., and McGUIRE, E. J. (2001) A brief history of testosterone, *The Journal of Urology 165*, 371-373.

47. Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., and Sumpter, J. P. (2001) Effects of the synthetic estrogen 17α‐ethinylestradiol on the life‐cycle of the fathead minnow (Pimephales promelas), *Environmental Toxicology and Chemistry 20*, 1216-1227.

48. Kwa, M., Plottel, C. S., Blaser, M. J., and Adams, S. (2016) The intestinal microbiome and estrogen receptor–positive female breast cancer, *JNCI: Journal of the National Cancer Institute 108*.

49. Magee, P. J., and Rowland, I. R. (2004) Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer, *British Journal of Nutrition 91*, 513-531.

50. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G. r., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.-Å. (2001) Mechanisms of estrogen action, *Physiological Reviews 81*, 1535-1565.

51. Campbell-Thompson, M., Lynch, I. J., and Bhardwaj, B. (2001) Expression of estrogen receptor (ER) subtypes and ERβ isoforms in colon cancer, *Cancer Research 61*, 632-640.

52. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., and Gustafsson, J.-A. k. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β, *Endocrinology 138*, 863-870.

53. Chen, K. L., and Madak-Erdogan, Z. (2016) Estrogen and microbiota crosstalk: should we pay attention?, *Trends in Endocrinology & Metabolism 27*, 752-755.

54. Ye, H., Dudley, S. Z., and Shaw, I. C. (2018) Intimate estrogen receptor-α/ligand relationships signal biological activity, *Toxicology*.

55. Balaguer, P., François, F., Comunale, F., Fenet, H., Boussioux, A.-M., Pons, M., Nicolas, J.-C., and Casellas, C. (1999) Reporter cell lines to study the estrogenic effects of xenoestrogens, *Science of the Total Environment 233*, 47-56.

56. Jensen, E. V., and DeSombre, E. R. (1972) Mechanism of action of the female sex hormones, *Annual Review of Biochemistry 41*, 203-230.

57. Roy, J. R., Chakraborty, S., and Chakraborty, T. R. (2009) Estrogen-like endocrine disrupting chemicals affecting puberty in humans--a review, *Medical Science Monitor 15*, RA137-RA145.

58. Iguchi, T., Katsu, Y., Horiguchi, T., Watanabe, H., Blumberg, B., and Ohta, Y. (2007) Endocrine disrupting organotin compounds are potent inducers of imposex in gastropods and adipogenesis in vertebrates, *Mol Cell Toxicol 3*, 1-10.

59. Lim, D., and Shaw, I. C. (2016) Is there a link between dietary phytoestrogens and reproductive health in men? A meta‐analysis of data from the USA and China, *International Journal of Food Science & Technology 51*, 23-29.

60. Dodds E C, G. L., Lawson W,. (1938) Oestrogenic activity of certain synthetic compounds, *Nature 141*, 247-248.

61. Sun, L., Yu, T., Guo, J., Zhang, Z., Hu, Y., Xiao, X., Sun, Y., Xiao, H., Li, J., and Zhu, D. (2016) The estrogenicity of methylparaben and ethylparaben at doses close to the acceptable daily intake in immature Sprague-Dawley rats, *Scientific Reports 6*.

62. McLACHLAN, J., Newbold, R. R., Burow, M. E., and Li, S. F. (2001) From malformations to molecular mechanisms in the male: three decades of research on endocrine disrupters, *Apmis 109*, S1-S11.

63. Degen, G., and Bolt, H. (2000) Endocrine disruptors: update on xenoestrogens, *International Archives of Occupational and Environmental Health 73*, 433-441.

64. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., Van Der Saag, P. T., Van Der Burg, B., and Gustafsson, J.-A. k. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β, *Endocrinology 139*, 4252-4263.

65. Wang, L.-Q. (2002) Mammalian phytoestrogens: enterodiol and enterolactone, *Journal of Chromatography B 777*, 289-309.

66. Bhathena, S. J., and Velasquez, M. T. (2002) Beneficial role of dietary phytoestrogens in obesity and diabetes, *The American Journal of Clinical Nutrition 76*, 1191-1201.

67. Cain, J. R., Lien, R. J., and Beasom, S. L. (1987) Phytoestrogen effects on reproductive performance of scaled quail, *The Journal of Wildlife Management*, 198-201.

68. Martin, P. M., Horwitz, K. B., Ryan, D. S., and Mcguire, W. L. (1978) Phytoestrogen interaction with estrogen receptors in human breast cancer cells, *Endocrinology 103*, 1860-1867.

69. Tassignon, J. l., and Haeseleer Abraham Borkowski, F. o. (1997) Natural antiestrogen receptor autoantibodies in man with estrogenic activity in mammary carcinoma cell culture: study of their mechanism of action; evidence for involvement of estrogen-like epitopes, *The Journal of Clinical Endocrinology & Metabolism 82*, 3464-3470.

70. Nikov, G. N., Hopkins, N. E., Boue, S., and Alworth, W. L. (2000) Interactions of dietary estrogens with human estrogen receptors and the effect on estrogen receptor-estrogen response element complex formation, *Environmental Health Perspectives 108*, 867.

71. Bickoff, E., Livingston, A., Hendrickson, A., and Booth, A. (1962) Forage estrogens, relative potencies of several estrogenlike compounds found in forages, *Journal of Agricultural and Food Chemistry 10*, 410-412.

72. Fang, H., Tong, W., Shi, L. M., Blair, R., Perkins, R., Branham, W., Hass, B. S., Xie, Q., Dial, S. L., and Moland, C. L. (2001) Structure− activity relationships for a large diverse set of natural, synthetic, and environmental estrogens, *Chemical Research in Toxicology 14*, 280-294.

73. Reinli, K., and Block, G. (1996) Phytoestrogen content of foods—a compendium of literature values, *Nutrition and Cancer 26*, 123-148.

74. Bickoff, E., Booth, A., Lyman, R., Livingston, A., Thompson, C., and Deeds, F. (1957) Coumestrol, a new estrogen isolated from forage crops, *Science (Washington) 126*, 969-970.

75. Kurzer, M. S., and Xu, X. (1997) Dietary phytoestrogens, *Annual Review of Nutrition 17*, 353-381.

76. Patisaul, H. B., Whitten, P. L., and Young, L. J. (1999) Regulation of estrogen receptor beta mRNA in the brain: opposite effects of 17β-estradiol and the phytoestrogen, coumestrol, *Molecular Brain Research 67*, 165-171.

77. Landete, J. (2012) Plant and mammalian lignans: a review of source, intake, metabolism, intestinal bacteria and health, *Food Research International 46*, 410-424.

78. Sirotkin, A. V., and Harrath, A. H. (2014) Phytoestrogens and their effects, *European Journal of Pharmacology 741*, 230-236.

79. Hu, C., Yuan, Y. V., and Kitts, D. D. (2007) Antioxidant activities of the flaxseed lignan secoisolariciresinol diglucoside, its aglycone secoisolariciresinol and the mammalian lignans enterodiol and enterolactone in vitro, *Food and Chemical Toxicology 45*, 2219-2227.

80. Wang, G., Kuan, S. S., Francis, O. J., Ware, G. M., and Carman, A. S. (1990) A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products, *Journal of Agricultural and Food Chemistry 38*, 185-190.

81. Salum, L. B., Polikarpov, I., and Andricopulo, A. D. (2008) Structure-based approach for the study of estrogen receptor binding affinity and subtype selectivity, *Journal of Chemical Information and Modeling 48*, 2243-2253.

82. Cassidy. (2003) Potential risks and benefits of phytoestrogen-rich diets, *International Journal for Vitamin and Nutrition Research 73*, 120-126.

83. Geisler, J., King, N., Dowsett, M., Ottestad, L., Lundgren, S., Walton, P., Kormeset, P., and Lønning, P. (1996) Influence of anastrozole (Arimidex), a selective, non-steroidal aromatase inhibitor, on in vivo aromatisation and plasma oestrogen levels in postmenopausal women with breast cancer, *British Journal of Cancer 74*, 1286.

84. Suthar, A., Banavalikar, M., and Biyani, M. (2001) Pharmacological activities of Genistein, an isoflavone from soy (Glycine max): Part II—Anti-cholesterol activity, effects on osteoporosis & menopausal symptoms.

85. Faber, K. A., and Hughes Jr, C. L. (1991) The effect of neonatal exposure to diethylstilbestrol, genistein, and zearalenone on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat, *Biology of Reproduction 45*, 649-653.

86. Whitten, P. L., Russell, E., and Naftolin, F. (1992) Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth, *Steroids 57*, 98-106.

87. Whitten, P. L., Lewis, C., and Naftolin, F. (1993) A phytoestrogen diet induces the premature anovulatory syndrome in lactationally exposed female rats, *Biology of Reproduction 49*, 1117-1121.

88. Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992) Evidence for decreasing quality of semen during past 50 years, *Bmj 305*, 609-613.

89. North, K., Golding, J., and Team, A. S. (2000) A maternal vegetarian diet in pregnancy is associated with hypospadias, *BJU international 85*, 107-113.

90. MacGillivray, M. H. (2004) Induction of puberty in hypogonadal children, *Journal of pediatric endocrinology & metabolism: JPEM 17*, 1277-1287.

91. Guillette Jr, L. J., Pickford, D. B., Crain, D. A., Rooney, A. A., and Percival, H. F. (1996) Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment, *General and Comparative Endocrinology 101*, 32-42.

92. Adlercreutz, H., Mousavi, Y., Clark, J., Höckerstedt, K., Hämäläinen, E., Wähälä, K., Mäkelä, T., and Hase, T. (1992) Dietary phytoestrogens and cancer: in vitro and in vivo studies, *The Journal of Steroid Biochemistry and Molecular Biology 41*, 331-337.

93. de Lemos, M. L. (2001) Effects of soy phytoestrogens genistein and daidzein on breast cancer growth, *Annals of Pharmacotherapy 35*, 1118-1121.

94. This, P., De La Rochefordi, A., Clough, K., Fourquet, A., and Magdelenat, H. (2001) Phytoestrogens after breast cancer, *Endocrine-related Cancer 8*, 129-134.

95. Peeters, P., Keinan-Boker, L., Van Der Schouw, Y., and Grobbee, D. (2003) Phytoestrogens and breast cancer risk, *Breast Cancer Research and Treatment 77*, 171-183.

96. Boué, S. M., Wiese, T. E., Nehls, S., Burow, M. E., Elliott, S., Carter-Wientjes, C. H., Shih, B. Y., McLachlan, J. A., and Cleveland, T. E. (2003) Evaluation of the estrogenic effects of legume extracts containing phytoestrogens, *Journal of Agricultural and Food Chemistry 51*, 2193-2199.

97. Wang, C., and Kurzer, M. S. (1997) Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells.

98. Lamartiniere, C. A., Moore, J., Holland, M., and Barnes, S. (1995) Neonatal genistein chemoprevents mammary cancer, *Proceedings of the Society for Experimental Biology and Medicine 208*, 120-123.

99. Cotterchio, M., Boucher, B. A., Manno, M., Gallinger, S., Okey, A., and Harper, P. (2006) Dietary phytoestrogen intake is associated with reduced colorectal cancer risk, *The Journal of Nutrition 136*, 3046-3053.

100. Knight, D. C., and Eden, J. A. (1996) A review of the clinical effects of phytoestrogens, *Obstetrics & Gynecology 87*, 897-904.

101. Newcomb, P. A., and Storer, B. E. (1995) Postmenopausal hormone use and risk of large-bowel cancer, *JNCI: Journal of the National Cancer Institute 87*, 1067-1071.

102. English, M. A., Kane, K. F., Cruickshank, N., Langman, M. J., Stewart, P. M., and Hewison, M. (1999) Loss of estrogen inactivation in colonic cancer, *The Journal of Clinical Endocrinology & Metabolism 84*, 2080-2085.

103. Martucci, C. P., and Fishman, J. (1993) P450 enzymes of estrogen metabolism, *Pharmacology & Therapeutics 57*, 237-257.

104. Kaldas, R. S., and Hughes Jr, C. L. (1989) Reproductive and general metabolic effects of phytoestrogens in mammals, *Reproductive Toxicology 3*, 81-89.

105. Whitten, P. L., Russell, E., and Naftolin, F. (1994) Influence of phytoestrogen diets on estradiol action in the rat uterus, *Steroids 59*, 443-449.

106. Hess, R. A., Fernandes, S. A., Gomes, G. R., Oliveira, C. A., Lazari, M. F., and Porto, C. S. (2011) Estrogen and its receptors in efferent ductules and epididymis, *Journal of Andrology 32*, 600-613.

107. Shaw, I. C. (2014) Chemical residues, food additives and natural toxicants in food–the cocktail effect, *International Journal of Food Science & Technology 49*, 2149-2157.

108. Lissin, L. W., and Cooke, J. P. (2000) Phytoestrogens and cardiovascular health, *Journal of the American College of Cardiology 35*, 1403-1410.

109. Honoré, E. K., Williams, J. K., Anthony, M. S., and Clarkson, T. B. (1997) Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques, *Fertility and Sterility 67*, 148-154.

110. Sullivan, J. M., and Fowlkes, L. P. (1996) Estrogens, menopause, and coronary artery disease, *Cardiology Clinics 14*, 105-116.

111. Dąbek, J., Kułach, A., and Gąsior, Z. (2010) Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB): a new potential therapeutic target in atherosclerosis?, *Pharmacological Reports 62*, 778-783.

112. Li, Y., Ellis, K.-L., Ali, S., El-Rayes, B. F., Nedeljkovic-Kurepa, A., Kucuk, O., Philip, P. A., and Sarkar, F. H. (2004) Apoptosis-inducing effect of chemotherapeutic agents is potentiated by soy isoflavone genistein, a natural inhibitor of NF-κB in BxPC-3 pancreatic cancer cell line, *Pancreas 28*, e90-e95.

113. Sakai, T., and Kogiso, M. (2008) Soy isoflavones and immunity, *The journal of Medical Investigation 55*, 167-173.

114. Vina, J., Gambini, J., Lopez-Grueso, R., M Abdelaziz, K., Jove, M., and Borras, C. (2011) Females live longer than males: role of oxidative stress, *Current Pharmaceutical Design 17*, 3959-3965.

115. Yellayi, S., Naaz, A., Szewczykowski, M. A., Sato, T., Woods, J. A., Chang, J., Segre, M., Allred, C. D., Helferich, W. G., and Cooke, P. S. (2002) The phytoestrogen genistein induces thymic and immune changes: a human health concern?, *Proceedings of the National Academy of Sciences 99*, 7616-7621.

116. Gorski, R. A. (1971) Gonadal hormones and the perinatal development of neuroendocrine function, *Frontiers in Neuroendocrinology 2*, 237-290.

117. Almey, A., Milner, T. A., and Brake, W. G. (2015) Estrogen receptors in the central nervous system and their implication for dopamine-dependent cognition in females, *Hormones and Behavior 74*, 125-138.

118. Gibbs, R. B. (1994) Estrogen and Nerve Growth Factor‐related Systems in Brain: Effects on Basal Forebrain Cholinergic Neurons and Implications for Learning and Memory Processes and Aging, *Annals of the New York Academy of Sciences 743*, 165-196.

119. Jacobs, E., and D'Esposito, M. (2011) Estrogen shapes dopamine-dependent cognitive processes: implications for women's health, *Journal of Neuroscience 31*, 5286-5293.

120. Yanagihara, N., Zhang, H., Toyohira, Y., Takahashi, K., Ueno, S., Tsutsui, M., and Takahashi, K. (2014) New insights into the pharmacological potential of plant flavonoids in the catecholamine system, *Journal of Pharmacological Sciences 124*, 123-128.

121. Hajirahimkhan, A., Dietz, B. M., and Bolton, J. L. (2013) Botanical modulation of menopausal symptoms: mechanisms of action?, *Planta Medica 79*, 538.

122. He, F.-J., and Chen, J.-Q. (2013) Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between Chinese women and women in Western countries and possible mechanisms, *Food Science and Human Wellness 2*, 146-161.

123. Franke, A. A., Custer, L. J., Cerna, C. M., and Narala, K. K. (1994) Quantitation of phytoestrogens in legumes by HPLC, *Journal of Agricultural and Food Chemistry 42*, 1905-1913.

124. Coward, L., Barnes, N. C., Setchell, K. D., and Barnes, S. (1993) Genistein, daidzein, and their. beta.-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets, *Journal of Agricultural and Food Chemistry 41*, 1961-1967.

125. Wang, H.-J., and Murphy, P. A. (1994) Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location, *Journal of Agricultural and Food Chemistry 42*, 1674-1677.

126. Qiu, L., and Chang, R. (2010) The origin and history of soybean, *The soybean: botany, production and uses*, 1-23.

127. Liu, K. (1997) Chemistry and nutritional value of soybean components, In *Soybeans*, pp 25-113, Springer.

128. Zambiazi, R. C., Przybylski, R., Zambiazi, M. W., and Mendonça, C. B. (2007) Fatty acid composition of vegetable oils and fats, *B. ceppa, curitiba 25*, 111-120.

129. Simopoulos, A. P., Leaf, A., and Salem Jr, N. (1999) Essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids, *Annals of Nutrition and Metabolism 43*, 127-130.

130. Anderson, J. W., Johnstone, B. M., and Cook-Newell, M. E. (1995) Meta-analysis of the effects of soy protein intake on serum lipids, *New England Journal of Medicine 333*, 276-282.

131. Nechuta, S. J., Caan, B. J., Chen, W. Y., Lu, W., Chen, Z., Kwan, M. L., Flatt, S. W., Zheng, Y., Zheng, W., and Pierce, J. P. (2012) Soy food intake after diagnosis of breast cancer and survival: an in-depth analysis of combined evidence from cohort studies of US and Chinese women–, *The American Journal of Clinical Nutrition 96*, 123-132.

132. Shurtleff, W., and Aoyagi, A. (2004) History of Soymilk and Dairy-like Soymilk Products. A Special Report on The History of Traditional Non-Fermented Soyfoods. A Chapter from the Unpublished Manuscript, *History of Soybeans and Soyfoods: 1100 BC to the 1980s*.

133. Madani, S., Prost, J., and Belleville, J. (2000) Dietary protein level and origin (casein and highly purified soybean protein) affect hepatic storage, plasma lipid transport, and antioxidative defense status in the rat, *Nutrition 16*, 368-375.

134. Washburn, S., Burke, G. L., Morgan, T., and Anthony, M. (1999) Effect of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women, *Menopause (New York, NY) 6*, 7-13.

135. Shurtleff, W., and Aoyagi, A. (2013) *History of tofu and tofu products (965 CE to 2013)*, Soyinfo Center.

136. Wang, H., and Cavins, J. (1989) Main content area Yield and amino acid composition of fractions obtained during tofu production, *Cereal Chemistry 66*, 359-361.

137. Saio, K., Kamiya, M., and Watanabe, T. (1969) Food processing characteristics of soybean 11s and 7s proteins: Part i. Effect of difference of protein components among soybean varieties on formation of tofu-gel, *Agricultural and Biological Chemistry 33*, 1301-1308.

138. Cai, T., and Chang, K. (1998) Characteristics of production-scale tofu as affected by soymilk coagulation method: propeller blade size, mixing time and coagulant concentration, *Food Research International 31*, 289-295.

139. Hirota, A., Taki, S., Kawaii, S., YANo, M., and Abe, N. (2000) 1, 1-Diphenyl-2-picrylhydrazyl radical-scavenging compounds from soybean miso and antiproliferative activity of isoflavones from soybean miso toward the cancer cell lines, *Bioscience, Biotechnology, and Biochemistry 64*, 1038-1040.

140. Shukla, S., Park, H.-K., Kim, J.-K., and Kim, M. (2011) Determination of biogenic amines in Japanese miso products, *Food Science and Biotechnology 20*, 851-854.

141. Shurtleff, W., and Aoyagi, A. (2012) *History of soy sauce (160 CE to 2012)*, Soyinfo Center.

142. Röling, W. F., Timotius, K. H., Prasetyo, A. B., Stouthamer, A. H., and Van Verseveld, H. W. (1994) Changes in microflora and biochemical composition during the baceman stage of traditional Indonesian kecap (soy sauce) production, *Journal of Fermentation and Bioengineering 77*, 62-70.

143. Fukushima, D. (2004) Industrialization of fermented soy sauce production centering around Japanese shoyu, *FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-*, 1-88.

144. Zhang, J. H., Tatsumi, E., Fan, J. F., and Li, L. T. (2007) Chemical components of Aspergillus‐type Douchi, a Chinese traditional fermented soybean product, change during the fermentation process, *International Journal of Food Science & Technology 42*, 263-268.

145. Moy, Y.-S., Lu, T.-J., and Chou, C.-C. (2012) Volatile components of the enzyme-ripened sufu, a Chinese traditional fermented product of soy bean, *Journal of Bioscience and Bioengineering 113*, 196-201.

146. Han, B.-Z., Rombouts, F. M., and Nout, M. R. (2001) A Chinese fermented soybean food, *International Journal of Food Microbiology 65*, 1-10.

147. Li-Jun, Y., Li-Te, L., Zai-Gui, L., Tatsumi, E., and Saito, M. (2004) Changes in isoflavone contents and composition of sufu (fermented tofu) during manufacturing, *Food Chemistry 87*, 587-592.

148. Kim, T.-W., Lee, J.-H., Kim, S.-E., Park, M.-H., Chang, H. C., and Kim, H.-Y. (2009) Analysis of microbial communities in doenjang, a Korean fermented soybean paste, using nested PCR-denaturing gradient gel electrophoresis, *International Journal of Food Microbiology 131*, 265-271.

149. Jung, B., and Roh, S. (2004) Physicochemical quality comparison of commercial doenjang and traditional green tea doenjang, *Journal of the Korean Society of Food Science and Nutrition*.

150. Kim, S.-H., and Lee, K.-A. (2003) Evaluation of taste compounds in water-soluble extract of a doenjang (soybean paste), *Food Chemistry 83*, 339-342.

151. Coward, L., Smith, M., Kirk, M., and Barnes, S. (1998) Chemical modification of isoflavones in soyfoods during cooking and processing, *The American Journal of Clinical Nutrition 68*, 1486S-1491S.

152. Kao, F.-J., Su, N.-W., and Lee, M.-H. (2004) Effect of water-to-bean ratio on the contents and compositions of isoflavones in tofu, *Journal of Agricultural and Food Chemistry 52*, 2277-2281.

153. Okubo, K., Kobayzshi, Y., and Takahashi, K. (1983) Improvement of soymilk and tofu process on the behavior of undesirable taste component such as glycosides, In *Food Proc*, pp 16-22.

154. Prabhakaran, M. P., Perera, C. O., and Valiyaveettil, S. (2006) Effect of different coagulants on the isoflavone levels and physical properties of prepared firm tofu, *Food Chemistry 99*, 492-499.

155. Esaki, H., Onozaki, H., Morimitsu, Y., Kawakishi, S., and Osawa, T. (1998) Potent antioxidative isoflavones isolated from soybeans fermented with Aspergillus saitoi, *Bioscience, Biotechnology, and Biochemistry 62*, 740-746.

156. Esaki, H., Kawakishi, S., Morimitsu, Y., and OSAWA, T. (1999) New potent antioxidative o-dihydroxyisoflavones in fermented Japanese soybean products, *Bioscience, Biotechnology, and Biochemistry 63*, 1637-1639.

157. Chang, T.-S. (2014) Isolation, bioactivity, and production of ortho-hydroxydaidzein and ortho-hydroxygenistein, *International Journal of Molecular Sciences 15*, 5699-5716.

158. Lee, D. E., Lee, K. W., Jung, S. K., Lee, E. J., Hwang, J. A., Lim, T.-G., Kim, B. Y., Bode, A. M., Lee, H. J., and Dong, Z. (2011) 6, 7, 4'-trihydroxyisoflavone inhibits HCT-116 human colon cancer cell proliferation by targeting CDK1 and CDK2, *Carcinogenesis*, bgr008.

159. Chang, T.-S., Ding, H.-Y., and Lin, H.-C. (2005) Identifying 6, 7, 4′-trihydroxyisoflavone as a potent tyrosinase inhibitor, *Bioscience, Biotechnology, and Biochemistry 69*, 1999-2001.

160. Lee, D. E., Lee, K. W., Jung, S. K., Lee, E. J., Hwang, J. A., Lim, T.-G., Kim, B. Y., Bode, A. M., Lee, H. J., and Dong, Z. (2011) 6, 7, 4′-trihydroxyisoflavone inhibits HCT-116 human colon cancer cell proliferation by targeting CDK1 and CDK2, *Carcinogenesis 32*, 629-635.

161. Chen, Y.-C., Inaba, M., Abe, N., and Hirota, A. (2003) Antimutagenic activity of 8-hydroxyisoflavones and 6-hydroxydaidzein from soybean miso, *Bioscience, Biotechnology, and Biochemistry 67*, 903-906.

162. Chang, T.-S., Ding, H.-Y., Tai, S. S.-K., and Wu, C.-Y. (2007) Mushroom tyrosinase inhibitory effects of isoflavones isolated from soygerm koji fermented with Aspergillus oryzae BCRC 32288, *Food Chemistry 105*, 1430-1438.

163. Sugiyama, Y., Sakurai, Y., and Hirota, A. (2010) Isolation of 2, 4, 4′-trihydroxydeoxybenzoin and 3′-hydroxydaidzein from soybean miso, *Bioscience, Biotechnology, and Biochemistry 74*, 1293-1294.

164. Kim, E. S., Shin, J. H., Seok, S. H., Kim, J. B., Chang, H., Park, S. J., Jo, Y. K., Choi, E. S., Park, J.-S., and Yeom, M. H. (2013) Autophagy mediates anti-melanogenic activity of 3′-ODI in B16F1 melanoma cells, *Biochemical and Biophysical Research Communications 442*, 165-170.

165. Lee, D. E., Lee, K. W., Song, N. R., Seo, S. K., Heo, Y.-S., Kang, N. J., Bode, A. M., Lee, H. J., and Dong, Z. (2010) 7, 3′, 4′-Trihydroxyisoflavone inhibits epidermal growth factor-induced proliferation and transformation of JB6 P+ mouse epidermal cells by suppressing cyclin-dependent kinases and phosphatidylinositol 3-kinase, *Journal of Biological Chemistry 285*, 21458-21466.

166. GYöRGY, P., MURATA, K., and IKEHATA, H. (1964) Antioxidants isolated from fermented soybeans (tempeh), *Nature 203*, 870.

167. Matsuda, H., Morikawa, T., Xu, F., Ninomiya, K., and Yoshikawa, M. (2004) New isoflavones and pterocarpane with hepatoprotective activity from the stems of Erycibe expansa, *Planta Medica 70*, 1201-1209.

168. Tewtrakul, S., Subhadhirasakul, S., Cheenpracha, S., and Karalai, C. (2007) HIV‐1 protease and HIV‐1 integrase inhibitory substances from Eclipta prostrata, *Phytotherapy Research 21*, 1092-1095.

169. Nguyen, D. T., Hernandez-Montes, E., Vauzour, D., Schönthal, A. H., Rice-Evans, C., Cadenas, E., and Spencer, J. P. (2006) The intracellular genistein metabolite 5, 7, 3′, 4′-tetrahydroxyisoflavone mediates G2-M cell cycle arrest in cancer cells via modulation of the p38 signaling pathway, *Free Radical Biology and Medicine 41*, 1225-1239.

170. Parkar, S. G., Trower, T. M., and Stevenson, D. E. (2013) Fecal microbial metabolism of polyphenols and its effects on human gut microbiota, *Anaerobe 23*, 12-19.

171. Glass, C. K., and Rosenfeld, M. G. (2000) The coregulator exchange in transcriptional functions of nuclear receptors, *Genes & Development 14*, 121-141.

172. Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving, *Endocrinology 132*, 2279-2286.

173. Meyers, M. J., Sun, J., Carlson, K. E., Marriner, G. A., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2001) Estrogen receptor-β potency-selective ligands: structure− activity relationship studies of diarylpropionitriles and their acetylene and polar analogues, *Journal of Medicinal Chemistry 44*, 4230-4251.

174. Graham, L., and Shaw, I. (2011) Does the oestrogen receptor encourage oestrogenicity in environmental pollutants? The case of 4-nonylphenol, *SAR and QSAR in Environmental Research 22*, 329-350.

175. Sonnenschein, C., and Soto, A. M. (1998) An updated review of environmental estrogen and androgen mimics and antagonists, *The Journal of Steroid Biochemistry and Molecular Biology 65*, 143-150.

176. Stender, J. D., Nwachukwu, J. C., Kastrati, I., Kim, Y., Strid, T., Yakir, M., Srinivasan, S., Nowak, J., Izard, T., and Rangarajan, E. S. (2017) Structural and Molecular Mechanisms of Cytokine-Mediated Endocrine Resistance in Human Breast Cancer Cells, *Molecular Cell 65*, 1122-1135. e1125.

177. Lannigan, D. A. (2003) Estrogen receptor phosphorylation, *Steroids 68*, 1-9.

178. Heldring, N., Pawson, T., McDonnell, D., Treuter, E., Gustafsson, J.-Å., and Pike, A. C. (2007) Structural insights into corepressor recognition by antagonist-bound estrogen receptors, *Journal of Biological Chemistry 282*, 10449-10455.

179. McInerney, E. M., Tsai, M.-J., O'Malley, B. W., and Katzenellenbogen, B. S. (1996) Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator, *Proceedings of the National Academy of Sciences 93*, 10069-10073.

180. Reid, G., Denger, S., Kos, M., and Gannon, F. (2002) Human estrogen receptor-α: regulation by synthesis, modification and degradation, *Cellular and Molecular Llife Sciences 59*, 821-831.

181. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000) Cofactor dynamics and sufficiency in estrogen receptor–regulated transcription, *Cell 103*, 843-852.

182. Müller, A., Makropoulos, V., and Bolt, H. (1995) Toxicological aspects of oestrogen-mimetic xenobiotics present in the environment, *Toxicol Ecotoxicol News 2*, 68-73.

183. Deroo, B. J., and Korach, K. S. (2006) Estrogen receptors and human disease, *The Journal of Clinical Investigation 116*, 561-570.

184. Van Lipzig, M. M., Ter Laak, A. M., Jongejan, A., Vermeulen, N. P., Wamelink, M., Geerke, D., and Meerman, J. H. (2004) Prediction of ligand binding affinity and orientation of xenoestrogens to the estrogen receptor by molecular dynamics simulations and the linear interaction energy method, *Journal of Medicinal Chemistry 47*, 1018-1030.

185. Siro, I., Kápolna, E., Kápolna, B., and Lugasi, A. (2008) Functional food. Product development, marketing and consumer acceptance—A review, *Appetite 51*, 456-467.

186. Ignatowicz, E., and Baer-Dubowska, W. (2001) Resveratrol, a natural chemopreventive agent against degenerative diseases, *Pol J Pharmacol 53*, 557-569.

187. Cook, N., and Samman, S. (1996) Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources, *The Journal of Nutritional Biochemistry 7*, 66-76.

188. Simons, L. A., von Konigsmark, M., Simons, J., and Celermajer, D. S. (2000) Phytoestrogens do not influence lipoprotein levels or endothelial function in healthy, postmenopausal women, *The American Journal of Cardiology 85*, 1297-1301.

189. Turner, N. J., Thomson, B. M., and Shaw, I. C. (2003) Bioactive isoflavones in functional foods: the importance of gut microflora on bioavailability, *Nutrition Reviews 61*, 204-213.

190. Breinholt, V., and Larsen, J. C. (1998) Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay, *Chemical Research in Toxicology 11*, 622-629.

191. Heinonen, S., Wähälä, K., and Adlercreutz, H. (1999) Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6′-OH-O-dma, and cis-4-OH-equol in human urine by gas chromatography–mass spectroscopy using authentic reference compounds, *Analytical Biochemistry 274*, 211-219.

192. Sirtris, G. (2011) A clinical study to assess the safety and activity of SRT501 alone or in combination with bortezomib in patients with multiple myeloma, *ClinicalTrials. gov [Online]. Bethesda (MD): National Library of Medicine (US). Available at: http://clinicaltrials. gov/show/NCT00920556 NLM Identifier: NCT00920556. Accessed on December 2*, 2011.

193. de Vos, P., Faas, M. M., Spasojevic, M., and Sikkema, J. (2010) Encapsulation for preservation of functionality and targeted delivery of bioactive food components, *International Dairy Journal 20*, 292-302.

194. Hall, L. M., Hall, L. H., and Kier, L. B. (2003) Modeling drug albumin binding affinity with e-state topological structure representation, *Journal of Chemical Information and Computer Sciences 43*, 2120-2128.

195. Ola, M. S., Nawaz, M., and Ahsan, H. (2011) Role of Bcl-2 family proteins and caspases in the regulation of apoptosis, *Molecular and Cellular Biochemistry 351*, 41-58.

196. Kitada, S., Leone, M., Sareth, S., Zhai, D., Reed, J. C., and Pellecchia, M. (2003) Discovery, characterization, and structure− activity relationships studies of

proapoptotic polyphenols targeting B-cell lymphocyte/leukemia-2 proteins, *Journal of Medicinal Chemistry 46*, 4259-4264.

197. Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2000) Pyrazole ligands: structure− affinity/activity relationships and estrogen receptor-α-selective agonists, *Journal of Medicinal Chemistry 43*, 4934-4947.

198. Li, F., Li, X., Shao, J., Chi, P., Chen, J., and Wang, Z. (2010) Estrogenic activity of anthraquinone derivatives: in vitro and in silico studies, *Chemical Research in Toxicology 23*, 1349-1355.

199. Ye, H., Dudley, S. Z., and Shaw, I. C. (2017) Escherichia coli biotransformation of daidzein fermentation products from soy‐based foods—relevance to food oestrogenicity‐based functionality, *International Journal of Food Science & Technology 52*, 1082-1091.

200. Jaga, K. (2000) What are the implications of the interaction between DDT and estrogen receptors in the body?, *Medical Hypotheses 54*, 18-25.

201. Frigo, D. E., Burow, M. E., Mitchell, K. A., Chiang, T.-C., and McLachlan, J. A. (2002) DDT and its metabolites alter gene expression in human uterine cell lines through estrogen receptor-independent mechanisms, *Environmental Health Perspectives 110*, 1239.

202. Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants, *Environmental Health Perspectives 103*, 113.

203. Mayr, U., Butsch, A., and Schneider, S. (1992) Validation of two in vitro test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts, *Toxicology 74*, 135-149.

204. Okubo, T., Yokoyama, Y., Kano, K., and Kano, I. (2001) ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERα and PR, *Food and Chemical Toxicology 39*, 1225-1232.

205. Zhu, B. T., Han, G.-Z., Shim, J.-Y., Wen, Y., and Jiang, X.-R. (2006) Quantitative structure-activity relationship of various endogenous estrogen metabolites for human

estrogen receptor α and β subtypes: Insights into the structural determinants favoring a differential subtype binding, *Endocrinology 147*, 4132-4150.

206. Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., and Perry, J. K. (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy, *Journal of Medicinal Chemistry 47*, 1739-1749.

207. Halgren, T. A., Murphy, R. B., Friesner, R. A., Beard, H. S., Frye, L. L., Pollard, W. T., and Banks, J. L. (2004) Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening, *Journal of Medicinal Chemistry 47*, 1750-1759.

208. Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., Sanschagrin, P. C., and Mainz, D. T. (2006) Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein− ligand complexes, *Journal of Medicinal Chemistry 49*, 6177-6196.

209. Manas, E. S., Xu, Z. B., Unwalla, R. J., and Somers, W. S. (2004) Understanding the selectivity of genistein for human estrogen receptor-β using X-ray crystallography and computational methods, *Structure 12*, 2197-2207.

210. Morito, K., Hirose, T., Kinjo, J., HIRAKAWA, T., OKAWA, M., NOHARA, T., OGAWA, S., INOUE, S., MURAMATSU, M., and MASAMUNE, Y. (2001) Interaction of phytoestrogens with estrogen receptors α and β, *Biological and Pharmaceutical Bulletin 24*, 351-356.

211. Turusov, V., Rakitsky, V., and Tomatis, L. (2002) Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks, *Environmental Health Perspectives 110*, 125.

212. Dixon, R. A. (2004) Phytoestrogens, *Annu. Rev. Plant Biol. 55*, 225-261.

213. Waring, R., and Harris, R. (2005) Endocrine disrupters: a human risk?, *Molecular and Cellular Endocrinology 244*, 2-9.

214. Harborne, J. B. (2013) *The flavonoids: advances in research since 1980*, Springer.

215. Ververidis, F., Trantas, E., Douglas, C., Vollmer, G., Kretzschmar, G., and Panopoulos, N. (2007) Biotechnology of flavonoids and other phenylpropanoid‐derived natural

products. Part I: Chemical diversity, impacts on plant biology and human health, *Biotechnology Journal 2*, 1214-1234.

216. Matsumoto, T., Kobayashi, M., Moriwaki, T., Kawai, S. i., and Watabe, S. (2004) Survey of estrogenic activity in fish feed by yeast estrogen-screen assay, *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 139*, 147-152.

217. Eldridge, M. D., Murray, C. W., Auton, T. R., Paolini, G. V., and Mee, R. P. (1997) Empirical scoring functions: I. The development of a fast empirical scoring function to estimate the binding affinity of ligands in receptor complexes, *Journal of Computer-aided Molecular Design 11*, 425-445.

218. Lin, S., Zhang, G., Liao, Y., Pan, J., and Gong, D. (2015) Dietary Flavonoids as Xanthine Oxidase Inhibitors: Structure–Affinity and Structure–Activity Relationships, *Journal of Agricultural and Food Chemistry 63*, 7784-7794.

219. Palusiak, M., and Grabowski, S. J. (2002) Methoxy group as an acceptor of proton in hydrogen bonds, *Journal of Molecular Structure 642*, 97-104.

220. Le Bail, J.-C., Champavier, Y., Chulia, A.-J., and Habrioux, G. (2000) Effects of phytoestrogens on aromatase, 3β and 17β-hydroxysteroid dehydrogenase activities and human breast cancer cells, *Life Sciences 66*, 1281-1291.

221. Anstead, G. M., Peterson, C. S., and Katzenellenbogen, J. A. (1989) Hydroxylated 2, 3-diarylindenes: Synthesis, estrogen receptor binding affinity, and binding orientation considerations, *Journal of Steroid Biochemistry 33*, 877-887.

222. Goyal, M., Rizzo, M., Schumacher, F., and Wong, C. F. (2009) Beyond thermodynamics: drug binding kinetics could influence epidermal growth factor signaling, *Journal of Medicinal Chemistry 52*, 5582-5585.

223. Pfitscher, A., Reiter, E., and Jungbauer, A. (2008) Receptor binding and transactivation activities of red clover isoflavones and their metabolites, *The Journal of Steroid Biochemistry and Molecular Biology 112*, 87-94.

224. Huntley, A. (2009) The health benefits of berry flavonoids for menopausal women: cardiovascular disease, cancer and cognition, *Maturitas 63*, 297-301.

225. Sharpe, R. M., Fisher, J. S., Millar, M. M., Jobling, S., and Sumpter, J. P. (1995) Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production, *Environmental Health Perspectives 103*, 1136.

226. Cutler Jr, G. (1997) The role of estrogen in bone growth and maturation during childhood and adolescence, *The Journal of Steroid Biochemistry and Molecular Biology 61*, 141-144.

227. Wolff, M. S., Britton, J. A., Boguski, L., Hochman, S., Maloney, N., Serra, N., Liu, Z., Berkowitz, G., Larson, S., and Forman, J. (2008) Environmental exposures and puberty in inner-city girls, *Environmental Research 107*, 393-400.

228. Ueno, T., and Uchiyama, S. (2001) Identification of the specific intestinal bacteria capable of metabolising soy isoflavone to equol, *Ann Nutr Metab 45*, 114.

229. Muthyala, R. S., Ju, Y. H., Sheng, S., Williams, L. D., Doerge, D. R., Katzenellenbogen, B. S., Helferich, W. G., and Katzenellenbogen, J. A. (2004) Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R-and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta, *Bioorganic & Medicinal Chemistry 12*, 1559-1567.

230. Shiau, A. K., Barstad, D., Radek, J. T., Meyers, M. J., Nettles, K. W., Katzenellenbogen, B. S., Katzenellenbogen, J. A., Agard, D. A., and Greene, G. L. (2002) Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism, *Nature Structural & Molecular Biology 9*, 359-364.

231. Fanning, S. W. (2017), RCSB PDB.

232. Mak, H. Y., Hoare, S., Henttu, P. M., and Parker, M. G. (1999) Molecular determinants of the estrogen receptor-coactivator interface, *Molecular and Cellular Biology 19*, 3895-3903.

233. Routledge, E. J., White, R., Parker, M. G., and Sumpter, J. P. (2000) Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) α and ERβ, *Journal of Biological Chemistry 275*, 35986-35993.

234. Shelby, M. D., Newbold, R. R., Tully, D. B., Chae, K., and Davis, V. L. (1996) Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays, *Environmental Health Perspectives 104*, 1296.

235. Pillon, A., Boussioux, A.-M., Escande, A., Aït-Aïssa, S., Gomez, E., Fenet, H., Ruff, M., Moras, D., Vignon, F., and Duchesne, M.-J. (2005) Binding of estrogenic compounds to recombinant estrogen receptor-α: application to environmental analysis, *Environmental Health Perspectives 113*, 278.

236. WHO, U. (2013) State of the science of endocrine disrupting chemicals 2012: an assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization, *Geneva, WHO*.

237. Höjer, A., Adler, S., Purup, S., Hansen-Møller, J., Martinsson, K., Steinshamn, H., and Gustavsson, A.-M. (2012) Effects of feeding dairy cows different legume-grass silages on milk phytoestrogen concentration, *Journal of Dairy Science 95*, 4526-4540.

238. Fang, H., Tong, W., Perkins, R., Soto, A. M., Prechtl, N. V., and Sheehan, D. M. (2000) Quantitative comparisons of in vitro assays for estrogenic activities, *Environmental Health Perspectives 108*, 723.

239. Odum, J., Lefevre, P., Tittensor, S., Paton, D., Routledge, E., Beresford, N., Sumpter, J., and Ashby, J. (1997) The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay, *Regulatory Toxicology and Pharmacology 25*, 176-188.

240. Legler, J., Zeinstra, L. M., Schuitemaker, F., Lanser, P. H., Bogerd, J., Brouwer, A., Vethaak, A. D., de Voogt, P., Murk, A. J., and van der Burg, B. (2002) Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity, *Environmental Science & Technology 36*, 4410-4415.

241. Berckmans, P., Leppens, H., Vangenechten, C., and Witters, H. (2007) Screening of endocrine disrupting chemicals with MELN cells, an ER-transactivation assay combined with cytotoxicity assessment, *Toxicology in Vitro 21*, 1262-1267.

242. Hoogenboom, L. A., De Haan, L., Hooijerink, D., Bor, G., Murk, A., and Brouwer, A. (2001) Estrogenic activity of estradiol and its metabolites in the ER‐CALUX assay with human T47D breast cells Note, *Apmis 109*, 101-107.

243. Hsieh, C.-Y., Santell, R. C., Haslam, S. Z., and Helferich, W. G. (1998) Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo, *Cancer Research 58*, 3833-3838.

244. Resende, F. A., de Oliveira, A. P. S., de Camargo, M. S., Vilegas, W., and Varanda, E. A. (2013) Evaluation of estrogenic potential of flavonoids using a recombinant yeast strain and MCF7/BUS cell proliferation assay, *Plos one 8*, e74881.

245. Zettner, A. (1973) Principles of competitive binding assays (saturation analyses). I. Equilibrium techniques, *Clinical Chemistry 19*, 699-705.

246. Korenman, S. G. (1969) Comparative binding affinity of estrogens and its relation to estrogenic potency, *Steroids 13*, 163-177.

247. Singh, K., Munuganti, R. S. N., Leblanc, E., Lin, Y. L., Leung, E., Lallous, N., Butler, M., Cherkasov, A., and Rennie, P. S. (2015) In silico discovery and validation of potent small-molecule inhibitors targeting the activation function 2 site of human oestrogen receptor α, *Breast Cancer Research 17*, 27.

248. Lorenzen, A., and Kennedy, S. W. (1993) A fluorescence-based protein assay for use with a microplate reader, *Analytical Biochemistry 214*, 346-348.

249. Rice-Evans, C. A., Miller, N. J., and Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids, *Free Radical Biology and Medicine 20*, 933-956.

250. Ekins, S., Mestres, J., and Testa, B. (2007) In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling, *British Journal of Pharmacology 152*, 9-20.

251. Wang, H.-j., and Murphy, P. A. (1994) Isoflavone content in commercial soybean foods, *Journal of Agricultural and Food Chemistry 42*, 1666-1673.

252. Dong, J.-Y., and Qin, L.-Q. (2011) Soy isoflavones consumption and risk of breast cancer incidence or recurrence: a meta-analysis of prospective studies, *Breast Cancer Research and Treatment 125*, 315-323.

253. Nazir, K. N. H., Ichinose, H., and Wariishi, H. (2010) Molecular characterization and isolation of cytochrome P450 genes from the filamentous fungus Aspergillus oryzae, *Archives of Microbiology 192*, 395-408.

254. Pandey, B. P., Lee, N., Choi, K.-Y., Jung, E., Jeong, D.-h., and Kim, B.-G. (2011) Screening of bacterial cytochrome P450s responsible for regiospecific hydroxylation of (iso) flavonoids, *Enzyme and Microbial Technology 48*, 386-392.

255. Hiller-Sturmhofel, S., and Bartke, A. (1998) The endocrine system: An overview, *Alcohol Health and Research World 22*, 153-164.

256. Mauvais-Jarvis, F., Clegg, D. J., and Hevener, A. L. (2013) The role of estrogens in control of energy balance and glucose homeostasis, *Endocrine Reviews 34*, 309-338.

257. Caballero, B. (2007) The global epidemic of obesity: an overview, *Epidemiologic Reviews 29*, 1-5.

258. Riant, E., Waget, A., Cogo, H., Arnal, J.-F., Burcelin, R., and Gourdy, P. (2009) Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice, *Endocrinology 150*, 2109-2117.

259. Böttner, M., Thelen, P., and Jarry, H. (2014) Estrogen receptor beta: tissue distribution and the still largely enigmatic physiological function, *The Journal of Steroid Biochemistry and Molecular Biology 139*, 245-251.

260. Mendelsohn, M. E., and Karas, R. H. (1999) The protective effects of estrogen on the cardiovascular system, *New England Journal of Medicine 340*, 1801-1811.

261. Murphy, E. (2011) Estrogen signaling and cardiovascular disease, *Circulation Research 109*, 687-696.

262. Skavdahl, M., Steenbergen, C., Clark, J., Myers, P., Demianenko, T., Mao, L., Rockman, H. A., Korach, K. S., and Murphy, E. (2005) Estrogen receptor-β mediates male-female differences in the development of pressure overload hypertrophy, *American Journal of Physiology-Heart and Circulatory Physiology 288*, H469-H476.

263. Roger, P., Sahla, M. E., Mäkelä, S., Gustafsson, J. Å., Baldet, P., and Rochefort, H. (2001) Decreased expression of estrogen receptor β protein in proliferative preinvasive mammary tumors, *Cancer research 61*, 2537-2541.

264. Baron, T. H., Ramirez, B., and Richter, J. E. (1993) Gastrointestinal motility disorders during pregnancy, *Annals of Internal Medicine 118*, 366-375.

265. Cheng, C., Chan, A. O. O., Hui, W. M., and Lam, S. K. (2003) Coping strategies, illness perception, anxiety and depression of patients with idiopathic constipation: a population‐based study, *Alimentary Pharmacology & Therapeutics 18*, 319-326.

266. Meier, R., Beglinger, C., Dederding, J., Meyer‐Wyss, B., Fumagalli, M., Rowedder, A., Turberg, Y., and Brignoli, R. (1995) Influence of age, gender, hormonal status and smoking habits on colonic transit time, *Neurogastroenterology & Motility 7*, 235-238.

267. Harnish, D. C., Albert, L. M., Leathurby, Y., Eckert, A. M., Ciarletta, A., Kasaian, M., and Keith Jr, J. C. (2004) Beneficial effects of estrogen treatment in the HLA-B27 transgenic rat model of inflammatory bowel disease, *American Journal of Physiology-Gastrointestinal and Liver Physiology 286*, G118-G125.

268. Looijer-van Langen, M., Hotte, N., Dieleman, L. A., Albert, E., Mulder, C., and Madsen, K. L. (2011) Estrogen receptor-β signaling modulates epithelial barrier function, *American Journal of Physiology-Gastrointestinal and Liver Physiology 300*, G621-G626.

269. Chen, J., Lin, H., and Hu, M. (2005) Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model, *Cancer Chemotherapy and Pharmacology 55*, 159-169.

270. Arai, N., Ström, A., Rafter, J. J., and Gustafsson, J.-Å. (2000) Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein, *Biochemical and Biophysical Research Communications 270*, 425-431.

271. Di Domenico, M., Castoria, G., Bilancio, A., Migliaccio, A., and Auricchio, F. (1996) Estradiol activation of human colon carcinoma-derived Caco-2 cell growth, *Cancer Research 56*, 4516-4521.

272. Budd, G. R., Aitchison, A., Day, A. S., and Keenan, J. I. (2017) The effect of polymeric formula on enterocyte differentiation, *Innate Immunity 23*, 240-248.

273. Kuntz, S., Wenzel, U., and Daniel, H. (1999) Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines, *European Journal of Nutrition 38*, 133-142.

274. Wakeling, A. E., and Bowler, J. (1992) ICI 182,780, a new antioestrogen with clinical potential, *The Journal of Steroid Biochemistry and Molecular Biology 43*, 173-177.

275. Forester, S. C., and Waterhouse, A. L. (2010) Gut metabolites of anthocyanins, gallic acid, 3-O-methylgallic acid, and 2, 4, 6-trihydroxybenzaldehyde, inhibit cell proliferation of Caco-2 cells, *Journal of Agricultural and Food Chemistry 58*, 5320-5327.

276. Chou, T.-C., and Talalay, P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, *Advances in Enzyme regulation 22*, 27-55.

277. Liu, Y., and Hu, M. (2002) Absorption and metabolism of flavonoids in the caco-2 cell culture model and a perused rat intestinal model, *Drug Metabolism and Disposition 30*, 370-377.

278. Hwang, E. Y., Huh, J.-W., Choi, M.-M., Choi, S. Y., Hong, H.-N., and Cho, S.-W. (2008) Inhibitory effects of gallic acid and quercetin on UDP‐glucose dehydrogenase activity, *FEBS letters 582*, 3793-3797.

279. Dornstauder, E., Jisa, E., Unterrieder, I., Krenn, L., Kubelka, W., and Jungbauer, A. (2001) Estrogenic activity of two standardized red clover extracts (Menoflavon®) intended for large scale use in hormone replacement therapy, *The Journal of Steroid Biochemistry and Molecular Biology 78*, 67-75.

280. Dalais, F., Rice, G., Wahlqvist, M., Grehar, M., Murkies, A., Medley, G., Ayton, R., and Strauss, B. (1998) Effects of dietary phytoestrogens in postmenopausal women, *Climacteric 1*, 124-129.

281. Tassignon, J., and Haeseleer Abraham Borkowski, F. o. (1997) Natural Antiestrogen Receptor Autoantibodies in Man with Estrogenic Activity in Mammary Carcinoma Cell Culture: Study of their Mechanism of Action; Evidence for Involvement of Estrogen-Like Epitopes 1, *The Journal of Clinical Endocrinology & Metabolism 82*, 3464-3470.

282. Heinonen, S.-M., Hoikkala, A., Wähälä, K., and Adlercreutz, H. (2003) Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects.: Identification of new metabolites having an intact isoflavonoid skeleton, *The Journal of Steroid Biochemistry and Molecular Biology 87*, 285-299.

283. Nakatsu, C. H., Armstrong, A., Clavijo, A. P., Martin, B. R., Barnes, S., and Weaver, C. M. (2014) Fecal bacterial community changes associated with isoflavone metabolites in postmenopausal women after soy bar consumption, *PloS one 9*, e108924.

284. Watanabe, S., Yamaguchi, M., Sobue, T., Takahashi, T., Miura, T., Arai, Y., Mazur, W., Wähälä, K., and Adlercreutz, H. (1998) Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako), *The Journal of Nutrition 128*, 1710-1715.

285. Atkinson, C., Frankenfeld, C. L., and Lampe, J. W. (2005) Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health, *Experimental Biology and Medicine 230*, 155-170.

286. Frankenfeld, C. L., Atkinson, C., Wähälä, K., and Lampe, J. W. (2014) Obesity prevalence in relation to gut microbial environments capable of producing equol or O-desmethylangolensin from the isoflavone daidzein, *European Journal of Clinical Nutrition 68*, 526-530.

287. Xu, Y., Nedungadi, T. P., Zhu, L., Sobhani, N., Irani, B. G., Davis, K. E., Zhang, X., Zou, F., Gent, L. M., and Hahner, L. D. (2011) Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction, *Cell Metabolism 14*, 453-465.

288. Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., and Batto, J.-M. (2011) Enterotypes of the human gut microbiome, *Nature 473*, 174.

289. Russell, D., Ross, R., Fitzgerald, G., and Stanton, C. (2011) Metabolic activities and probiotic potential of bifidobacteria, *International Journal of Food Microbiology 149*, 88-105.

290. Cani, P. D., and Delzenne, N. M. (2009) The role of the gut microbiota in energy metabolism and metabolic disease, *Current Pharmaceutical Design 15*, 1546-1558.

291. Duncan, S. H., Belenguer, A., Holtrop, G., Johnstone, A. M., Flint, H. J., and Lobley, G. E. (2007) Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces, *Applied and Environmental Microbiology 73*, 1073-1078.

292. Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L. G., Gratadoux, J.-J., Blugeon, S., Bridonneau, C., Furet, J.-P., and Corthier, G. (2008) Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients, *Proceedings of the National Academy of Sciences 105*, 16731-16736.

293. Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012) The impact of the gut microbiota on human health: an integrative view, *Cell 148*, 1258-1270.

294. De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., and Lionetti, P. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa, *Proceedings of the National Academy of Sciences 107*, 14691-14696.

295. Musso, G., Gambino, R., and Cassader, M. (2010) Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded?, *Diabetes care 33*, 2277-2284.

296. Heaver, S. L., Johnson, E. L., and Ley, R. E. (2018) Sphingolipids in host–microbial interactions, *Current Opinion in Microbiology 43*, 92-99.

297. Murphy, E., Cotter, P., Healy, S., Marques, T. M., O'sullivan, O., Fouhy, F., Clarke, S., O'toole, P., Quigley, E. M., and Stanton, C. (2010) Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models, *Gut*, gut. 2010.215665.

298. Krajmalnik‐Brown, R., Ilhan, Z. E., Kang, D. W., and DiBaise, J. K. (2012) Effects of gut microbes on nutrient absorption and energy regulation, *Nutrition in Clinical Practice 27*, 201-214.

299. Wang, S.-T., Fang, T.-F., Hsu, C., Chen, C.-H., Lin, C.-J., and Su, N.-W. (2015) Biotransformed product, genistein 7-O-phosphate, enhances the oral bioavailability of genistein, *Journal of Functional Foods 13*, 323-335.

300. Parkar, S. G., Simmons, L., Herath, T. D., Phipps, J. E., Trower, T. M., Hedderley, D. I., McGhie, T. K., Blatchford, P., Ansell, J., and Sutton, K. H. (2018) Evaluation of the prebiotic potential of five kiwifruit cultivars after simulated gastrointestinal digestion and fermentation with human faecal bacteria, *International Journal of Food Science & Technology 53*, 1203-1210.

301. Candela, M., Maccaferri, S., Turroni, S., Carnevali, P., and Brigidi, P. (2010) Functional intestinal microbiome, new frontiers in prebiotic design, *International Journal of Food microbiology 140*, 93-101.

302. Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., and Tanaka, R. (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces, *Applied and Environmental Microbiology 70*, 7220-7228.

303. Hubatsch, I., Ragnarsson, E. G., and Artursson, P. (2007) Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers, *Nature Protocols 2*, 2111.

304. Chen, J., Lin, H., and Hu, M. (2003) Metabolism of flavonoids via enteric recycling: role of intestinal disposition, *Journal of Pharmacology and Experimental Therapeutics 304*, 1228-1235.

305. O'Mahony, L., McCarthy, J., Kelly, P., Hurley, G., Luo, F., Chen, K., O'Sullivan, G. C., Kiely, B., Collins, J. K., and Shanahan, F. (2005) Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles, *Gastroenterology 128*, 541-551.

306. Eppinga, H., Sperna Weiland, C. J., Thio, H. B., van der Woude, C. J., Nijsten, T. E., Peppelenbosch, M. P., and Konstantinov, S. R. (2016) Similar depletion of protective Faecalibacterium prausnitzii in psoriasis and inflammatory bowel disease, but not in hidradenitis suppurativa, *Journal of Crohn's and Colitis 10*, 1067-1075.

307. Sommer, F., and Bäckhed, F. (2013) The gut microbiota—masters of host development and physiology, *Nature Reviews Microbiology 11*, 227.

308. Cani, P. D., and Everard, A. (2016) Talking microbes: when gut bacteria interact with diet and host organs, *Molecular Nutrition & Food Research 60*, 58-66.

309. Sheridan, W. G., Lowndes, R. H., and Young, H. L. (1990) Intraoperative tissue oximetry in the human gastrointestinal tract, *The American Journal of Surgery 159*, 314-319.

310. Britton, R., and Krehbiel, C. (1993) Nutrient Metabolism by Gut Tissues1, *Journal of Dairy Science 76*, 2125-2131.

311. Izumi, T., Piskula, M. K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y., and Kikuchi, M. (2000) Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans, *The Journal of Nutrition 130*, 1695-1699.

312. Dueñas, M., Muñoz-González, I., Cueva, C., Jiménez-Girón, A., Sánchez-Patán, F., Santos-Buelga, C., Moreno-Arribas, M., and Bartolomé, B. (2015) A survey of modulation of gut microbiota by dietary polyphenols, *BioMed Research International 2015*.

313. Wu, B., Basu, S., Meng, S., Wang, X., and Hu, M. (2011) Regioselective sulfation and glucuronidation of phenolics: insights into the structural basis, *Current Drug Metabolism 12*, 900-916.

314. Bennink, M. R. (2010) Dietary soy reduces colon carcinogenesis in human and rats, In *Nutrition and Cancer Prevention*, pp 11-17, Springer.

315. Wong, W. M., and Wright, N. A. (1999) Cell proliferation in gastrointestinal mucosa, *Journal of Clinical Pathology 52*, 321-333.

316. Gillies, G. E., and McArthur, S. (2010) Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines, *Pharmacological Reviews 62*, 155-198.

317. Zhu, L., Yang, Y., Xu, P., Zou, F., Yan, X., Liao, L., Xu, J., O'Malley, B. W., and Xu, Y. (2013) Steroid receptor coactivator-1 mediates estrogenic actions to prevent body weight gain in female mice, *Endocrinology 154*, 150-158.

318. Potter, S. M., Baum, J. A., Teng, H., Stillman, R. J., Shay, N. F., and Erdman Jr, J. W. (1998) Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women, *The American Journal of Clinical Nutrition 68*, 1375S-1379S.

319. Ju, Y. H., Doerge, D. R., Allred, K. F., Allred, C. D., and Helferich, W. G. (2002) Dietary genistein negates the inhibitory effect of tamoxifen on growth of estrogen-dependent human breast cancer (MCF-7) cells implanted in athymic mice, *Cancer Research 62*, 2474-2477.

320. Guo, J., Xiao, B., Liu, D., Grant, M., Zhang, S., Lai, Y., Guo, Y., and Liu, Q. (2004) Biphasic effect of daidzein on cell growth of human colon cancer cells, *Food and Chemical Toxicology 42*, 1641-1646.

321. Ávila-Gálvez, M. Á., Espín, J. C., and González-Sarrías, A. (2018) Physiological Relevance of the Antiproliferative and Estrogenic Effects of Dietary Polyphenol Aglycones versus Their Phase-II Metabolites on Breast Cancer Cells: A Call of Caution, *Journal of Agricultural and Food Chemistry 66*, 8547-8555.

322. Izgelov, D., Cherniakov, I., Aldouby Bier, G., Domb, A. J., and Hoffman, A. (2018) The effect of piperine pro-nano lipospheres on direct intestinal phase II metabolism: the

raloxifene paradigm of enhanced oral bioavailability, *Molecular Pharmaceutics 15*, 1548-1555.

323. Murota, K., Nakamura, Y., and Uehara, M. (2018) Flavonoid metabolism: The interaction of metabolites and gut microbiota, *Bioscience, Biotechnology, and Biochemistry 82*, 600-610.

324. Liu, J. Y., Lin, G., Fang, M., and Rudd, J. A. (2019) Localization of estrogen receptor ERα, ERβ and GPR30 on myenteric neurons of the gastrointestinal tract and their role in motility, *General and Comparative Endocrinology 272*, 63-75.

325. Nie, X., Xie, R., and Tuo, B. (2018) Effects of estrogen on the gastrointestinal tract, *Digestive Diseases and Sciences*, 1-14.

326. Chang, Y.-C., and Nair, M. G. (1995) Metabolism of daidzein and genistein by intestinal bacteria, *Journal of Natural Products 58*, 1892-1896.

327. Loftus, E. V., Silverstein, M. D., Sandborn, W. J., Tremaine, W. J., Harmsen, W. S., and Zinsmeister, A. R. (1998) Crohn's disease in Olmsted County, Minnesota, 1940–1993: incidence, prevalence, and survival, *Gastroenterology 114*, 1161-1168.

328. Lapidus, A., Bernell, O., Hellers, G., Persson, P., and Löfberg, R. (1997) Incidence of Crohn's disease in Stockholm County 1955–1989, *Gut 41*, 480-486.

329. Lesko, S. M., Kaufman, D. W., Rosenberg, L., Helmrich, S. P., Miller, D. R., Stolley, P. D., and Shapiro, S. (1985) Evidence for an increased risk of Crohn's disease in oral contraceptive users, *Gastroenterology 89*, 1046-1049.

330. Bryzgalova, G., Gao, H., Ahrén, B., Zierath, J., Galuska, D., Steiler, T., Dahlman-Wright, K., Nilsson, S., Gustafsson, J.-Å., and Efendic, S. (2006) Evidence that oestrogen receptor-α plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver, *Diabetologia 49*, 588-597.

331. Barratt, M. J., Lebrilla, C., Shapiro, H.-Y., and Gordon, J. I. (2017) The gut microbiota, food science, and human nutrition: a timely marriage, *Cell Host & Microbe 22*, 134-141.

332. Okubo, K., KOBAYASHI, K., and Takahashi, K. (1983) Improvement of soymilk and tofu process on the behavior of undesirable taste component such as glycosides, *Up-To-Date Food Process.(Japan) 18*, 16-22.

333. Kang, S., Denman, S. E., Morrison, M., Yu, Z., Dore, J., Leclerc, M., and McSweeney, C. S. (2010) Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray, *Inflammatory Bowel Diseases 16*, 2034-2042.

334. Kemperman, R. A., Bolca, S., Roger, L. C., and Vaughan, E. E. (2010) Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities, *Microbiology 156*, 3224-3231.

335. Wang, H., Wei, C.-X., Min, L., and Zhu, L.-Y. (2018) Good or bad: gut bacteria in human health and diseases, *Biotechnology & Biotechnological Equipment*, 1-6.

336. Liang, D., Leung, R. K.-K., Guan, W., and Au, W. W. (2018) Involvement of gut microbiome in human health and disease: brief overview, knowledge gaps and research opportunities, *Gut Pathogens 10*, 3.

337. Schmidt, C. (2015) Mental health: thinking from the gut, *Nature 518*, S12-S15.

338. Bienenstock, J., Kunze, W. A., and Forsythe, P. (2018) Disruptive physiology: olfaction and the microbiome–gut–brain axis, *Biological Reviews 93*, 390-403.

339. Osadchiy, V., Martin, C. R., and Mayer, E. A. (2018) The Gut-Brain Axis and the Microbiome: Mechanisms and Clinical Implications, *Clinical Gastroenterology and Hepatology*.

340. Dalile, B., Van Oudenhove, L., Verbeke, K., and Vervliet, B. (2018) Nourishing the gut microbiota: The potential of prebiotics in microbiota-gut-brain axis research, *Behavioral and Brain Sciences*.

341. Agustí, A., García-Pardo, M. P., López-Almela, I., Campillo, I., Maes, M., Romaní-Pérez, M., and Sanz, Y. (2018) Interplay between the gut-brain axis, obesity and cognitive function, *Frontiers in Neuroscience 12*, 155.

342. Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., and Palva, A. (2004) Development of an extensive set of 16S rDNA‐targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real‐time PCR, *Journal of Applied Microbiology 97*, 1166-1177.

343. Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., and Wang, K. (2008) Development of a real‐time PCR method for Firmicutes and Bacteroidetes in faeces and its application

to quantify intestinal population of obese and lean pigs, *Letters in Applied Microbiology 47*, 367-373.

344. Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., and Louis, P. (2008) Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii, *British Journal of Nutrition 101*, 541-550.

345. Paturi, G., Butts, C. A., Bentley‐Hewitt, K. L., and Ansell, J. (2014) Influence of green and gold kiwifruit on indices of large bowel function in healthy rats, *Journal of Food Science 79*, H1611-H1620.

Appendix

Appendix

Appendix A: Supporting information for Chapter 3

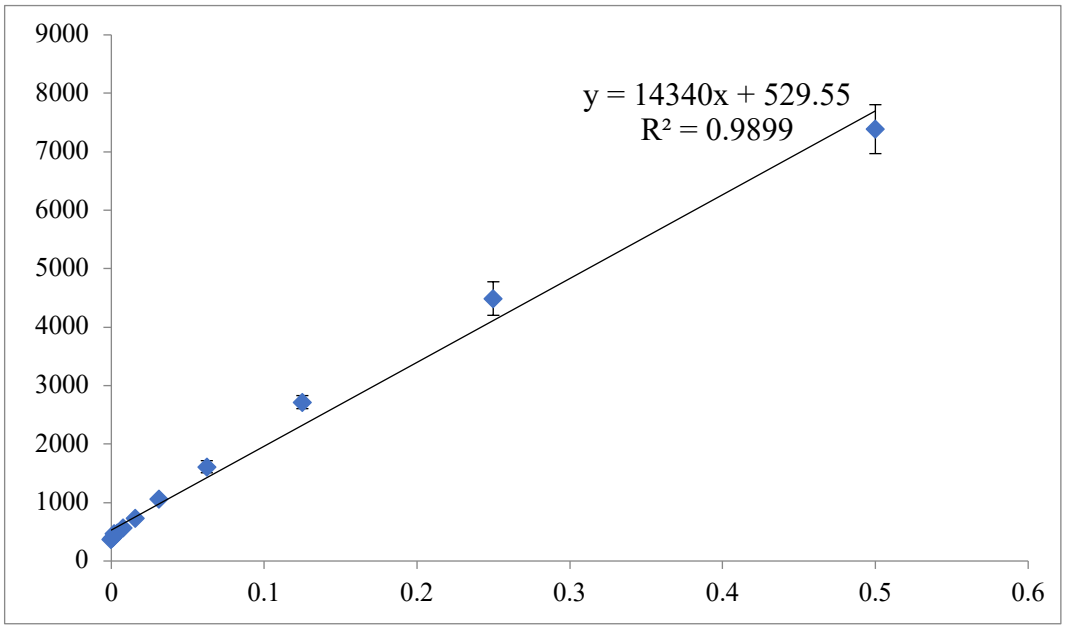
Table S 1: Missing amino acid residues of the complexes of ERα with different ligands.

PDB code	Missing amino acid residues
1ERE	SER 301, LYS 302, LYS 303, ASN 304, TYR 331, ASP 332, PRO 333, THR 334, ARG 335, PRO 336, LEU 462, SER 463, SER 464, LEU 549, HIS 550, ALA 551, PRO 552, THR 553
1ERR	SER 301, LYS 302, LYS 303, ASN 304, SER 305, LEU 306, THR 460, PHE 461, LEU 462, SER 463, SER 464, THR 465, LEU 466, LYS 467, SER 468, LEU 469, LYS 529, CYS 530,
1X7R	SER 305, TYR 331, ASP 332, PRO 333, THR 334, ARG 335, PRO 336, PHE 337, ARG 548, LEU 549, LYS 686
4TV1	LYS 302, LYS 303, ASN 304, LEU 462, SER 463, SER 464, THR 465, LEU 466, LYS 467, SER 468, LEU 469, LEU 549, HIS 550, ALA 551, PRO 552

5T1Z	LEU 462, SER 463, SER 464, THR 465, LEU 466, HIS 550, ALA 551, PRO 552, THR 553, SER 554
5U2D	ILE 298, LYS 299, ARG 300, SER 301, LYS 302, LYS 303, ASN 304, LEU 462, SER 463, SER 464, SER 554

Appendix B: Supporting information for Chapter 4

Figure S 1: Calibration graph of the protein content in MELN cells (exposure to isoflavones) in the fluorescence assay (n = 3).

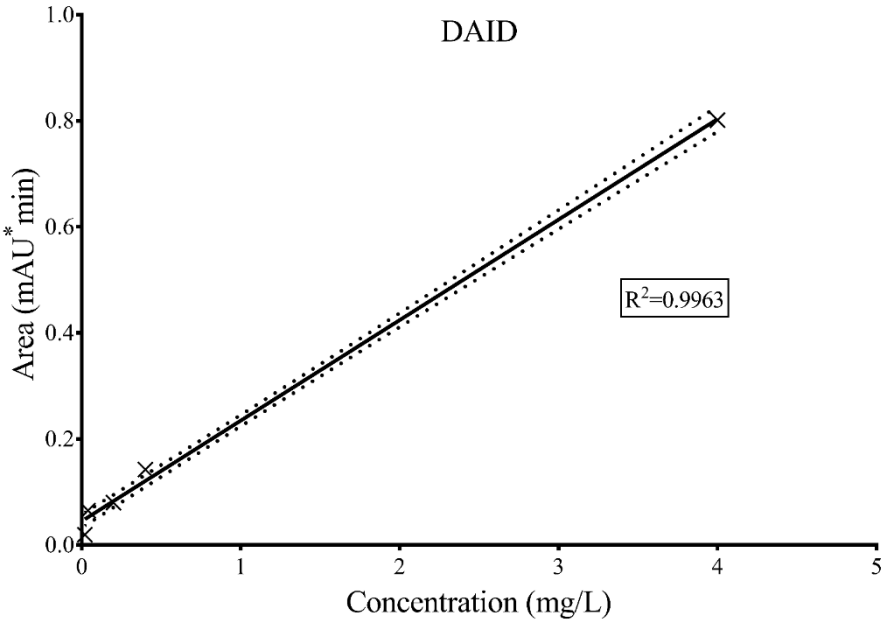


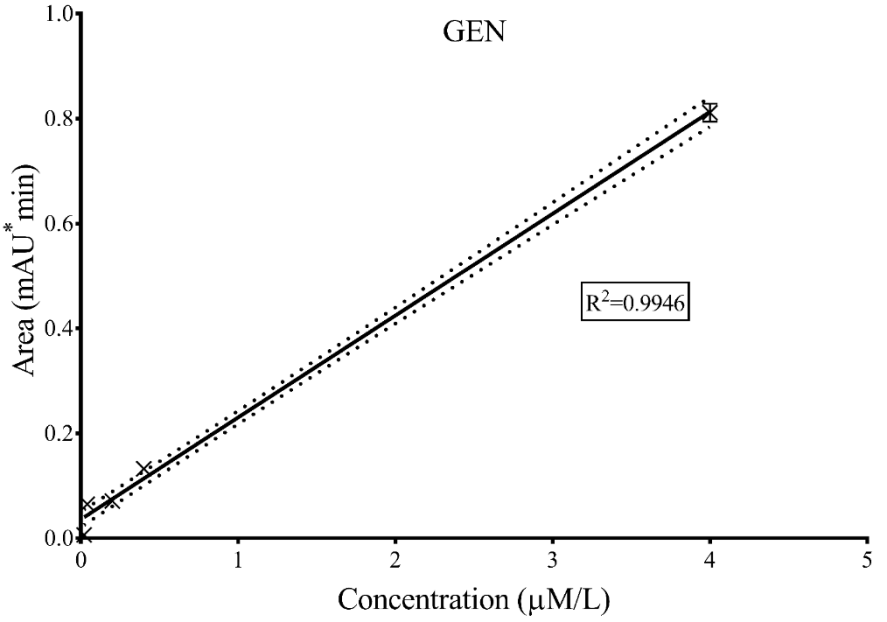
Appendix C: Supporting information for Chapter 6

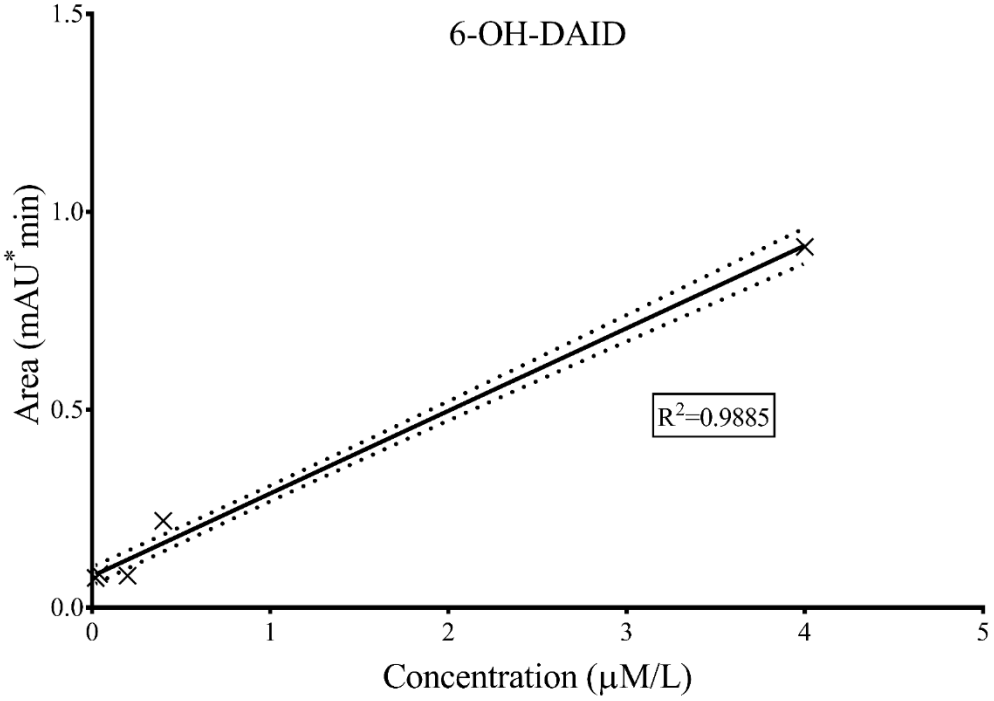
Table S 2: The bacterial standards, primers and the annealing conditions used for real time PCR quantification of bacteria

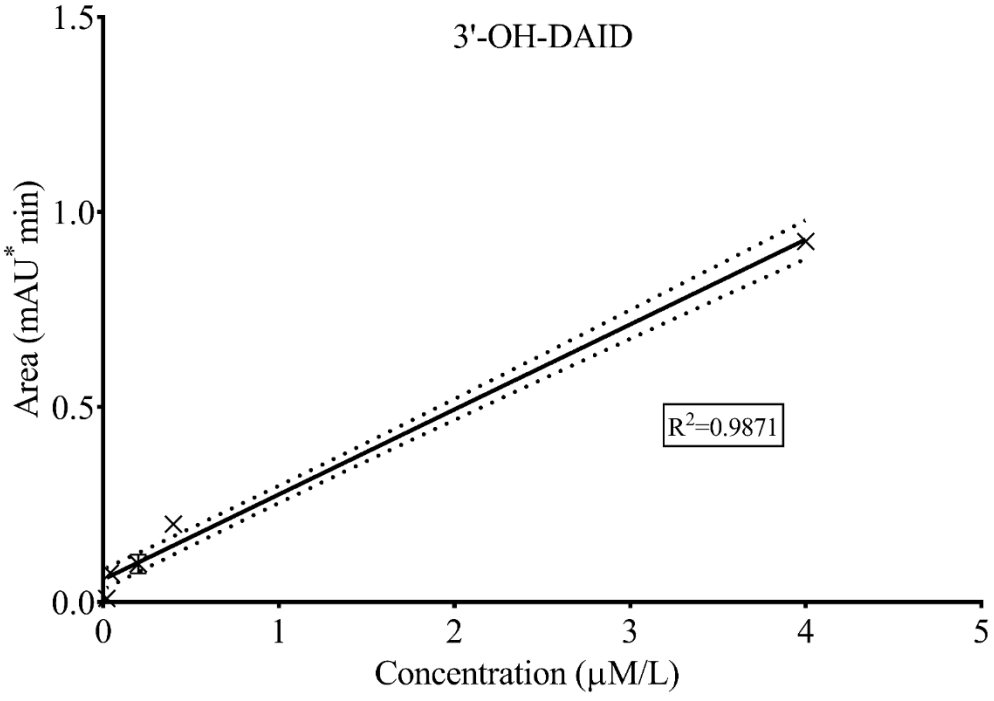
Target bacteria	Bacterial standard	Primer sequence (5´ → 3´)	Temperature and time for
Bacteroides-Prevotella-Porphyromonas	*Bacteroides thetaiotamicron* ATCC 29148	Fwd: GGTGTCGGCTTAAGTGCCAT	63 °C, 20 s [342]
Firmicutes	*Lactobacillus rhamnosus* ATCC 7469	Fwd: GGAGYATGTGGTTTAATTCGAAGCA	60 °C, 20 s [343]
Faecalibacterium prausnitzii	*F. prausnitzii* DSM 17677	Fwd: GGAGGAAGAAGGTCTTCGG	60 °C, 20 s [344]
***Bifidobacterium* spp.**	*Bifidobacterium longum* ATCC 15707	Fwd: GGGTGGTAATGCCGGATG	66 °C, 45 s [345]

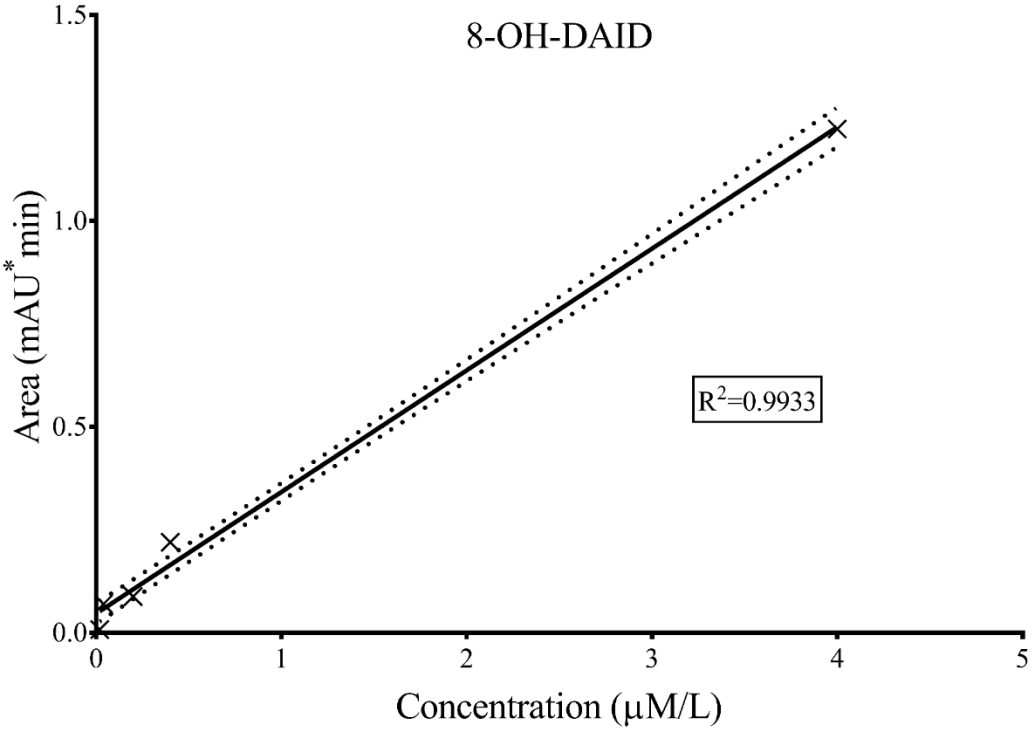
Figure S 2: Calibration graphs for all selected isoflavones authentic standards. All show good linear regression with R2 values in the range of 0.98–0.99 (n = 3).

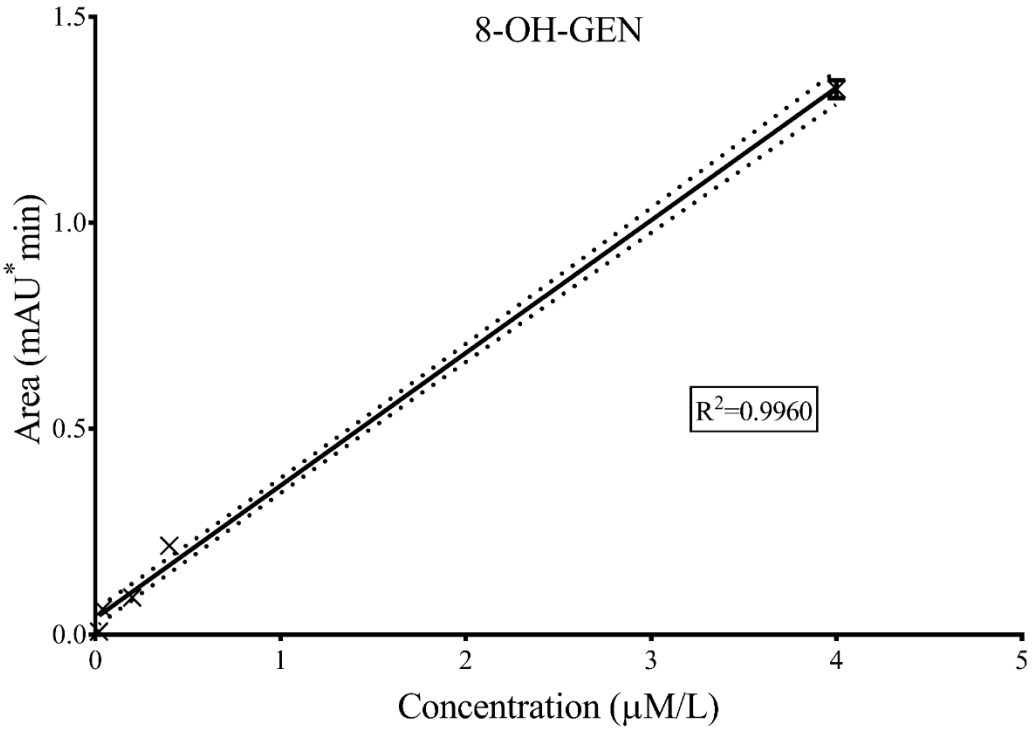





6-OH-DAID
Area (mAU*min)
Concentration (μM/L)
R2=0.9885
0.0
0.5
1.0
1.5
0
1
2
3
4
5


3'-OH-DAID
Area (mAU*min)
Concentration (μM/L)
R2=0.9871
0.0
0.5
1.0
1.5
0
1
2
3
4
5


8-OH-DAID
Area (mAU*min)
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Concentration (μM/L)
R2=0.9933


8-OH-GEN
Area (mAU*min)
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Concentration (μM/L)
R2=0.9960

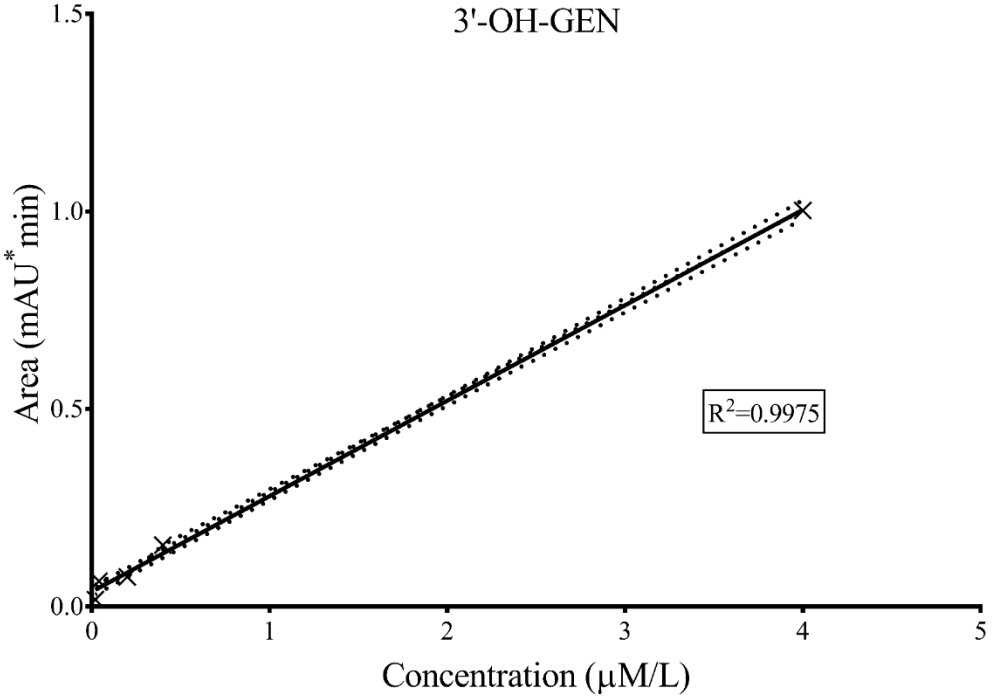

3'-OH-GEN
Area (mAU* min)
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Concentration (µM/L)
R2=0.9975

Table S 3: The effects of selected isoflavones on the growth of Firmicutes, Bacteriodetes and thus the F/B ratio

Compound	Time (h)	Firmicutes (F)	Bacteriodetes (B)
Control (no isoflavones)	0	10.1709 ± 0.0143	6.4069 ± 0.0074
	2	9.9241±0.0056	5.6606 ± 0.0107
	20	9.541± 0.0178	4.5374 ± 0.0210
Inulin	2	10.1889±0.0202	6.5280 ± 0.0078
	20	10.2535±0.0217	6.84306 ± 0.0049
DAID	2	9.91185±0.0097	6.9446 ± 0.0087
	20	8.9749±0.0110	6.0260 ± 0.0108
6-OH-DAID	2	9.5322±0.1770	6.6530 ± 0.0167
	20	9.3306 ± 0.1801	6.082592476 ± 0.0043
3′-OH-DAID	2	9.8320 ± 0.0455	7.177476922 ± 0.0059
	20	9.4818 ± 0.0698	6.528309519 ± 0.0113
8-OH-DAID	2	9.7851 ± 0.1015	6.371815434 ± 0.0077

	20	8.7884 ± 0.1980	5.561316933 ± 0.0108
GEN	2	10.1157 ± 0.0076	7.22246626 ± 0.0033
	20	9.1790 ± 0.1006	5.91983807 ± 0.0201
3′-OH-GEN	2	9.0846 ± 0.0945	6.639486 ± 0.0191
	20	9.7550 ± 0.07876	6.322553162 ± 0.0452
8-OH-GEN	2	9.8386 ± 0.1015	6.37963775 ± 0.0390
	20	9.3170 ± 0.1446	5.025852 ± 0.0177

Appendix D: Publications from this thesis

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Review

Intimate estrogen receptor-α/ligand relationships signal biological activity

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ABSTRACT

How does estrogen receptor-α bind its natural ligands – estrogens? How can other molecules mimic estrogens and elicit different estrogenic responses? The answers lie in a complex and intimate chemical biology between ligands and receptor. This delicate interaction at the ligand binding cleft signals, via conformational change, exposure of a specific new charge topography at a second site (Activation Function-2). This, in turn, attracts a regulatory protein which modulates gene expression and controls biological activity.

1. What do we know about estrogen receptors?

Estrogen receptors (ERs) have functions way beyond traditional ideas of estrogen activity. Not only do they initiate and guide sexual development, they also have key roles in stimulating cell division; for example, early neurological development (Gillies and McArthur, 2010; Heldring et al., 2007b). All of this is achieved by a specialised region of the receptor (binding cleft) which interacts with estrogens to cause conformational changes in the receptor which in turn leads to receptor/ligand complex dimerization followed by occupancy of a DNA region (Estrogen Responsive Element - ERE) which controls gene expression of key genes (Fig. 1) (Shiau et al., 1998).

There are two estrogen receptor isoforms α and β. Structurally they are similar, but their cellular effects are significantly different, perhaps because of their different cell distributions – e.g., breast cancer cells express mainly ERα, whereas gut cells express mainly ERβ (Arai et al., 2000; Okubo et al., 2001). We will focus on ERα in this article because it is the most studied of the receptor isoforms because of its clinical significance (e.g. in breast cancer). ERα is a 17β-estradiol (E2)-activated nuclear receptor (NR3A1 - nuclear receptor subfamily 3, group A, member 1) with remarkably broad ranging effects mediated by occupancy of its ligand binding domain (LBD). ERs have six domains (A–F) including three major functional domains comprising an N-terminal domain which hosts a transcriptional activation function (AF-1), a DNA-binding domain, and a C-terminal LBD (Delfosse et al., 2014; Klinge, 2001).

The LBD comprises two separate, but interacting binding clefts – the ligand binding cleft (LBC) and Activation Function-2 (AF-2) (Brzozowski et al., 1997). Molecular interplay between LBC and AF-2 occupancy determines ERα activity. The LBC binds a ligand (either agonist or antagonist) which initiates a conformational change which exposes AF-2 to allow its interaction with regulatory proteins (Brzozowski et al., 1997). The conformational change initiated by ligand binding initiates ERα dissociation from a heat shock protein (usually Hsp90). Phosphorylation then occurs which aids receptor dimerization. The dimer then moves into the nucleus and binds to DNA via the ERE or via a protein DNA binding intermediate (Murphy et al., 2011). Coregulatory protein recruitment then occurs (Fig. 1).(Shiau et al., 1998). In addition, the coregulatory proteins comprise coactivators (promotors of estrogenicity) and corepressors (suppressors of ER activity). The bound regulatory protein establishes a "triangular relationship" with the ER and the bound ligand (Katzenellenbogen and Katzenellenbogen, 2002) which facilitates fine tuning of the estrogenic response. Furthermore, differential coregulatory protein recruitment contributes to the tissue-specific effects of selective ER modulators (SERMs) (Heldring et al., 2007a).

Occupancy of the LBD can lead to either agonism or antagonism of ERα activity. These two modes of activity are likely to be determined by the manner and strength of binding of ligands to the LBC. For example, a ligand that binds strongly, but does not interact with amino acid residues in a manner that facilitates the receptor conformational change that leads to its interaction with DNA might inhibit E2's agonistic activity - i.e. it blocks the LBC. A good example of an ERα antagonist is 4-hydroxytamoxifen (a cytochrome-P450 catalysed metabolite of the anti-breast cancer drug tamoxifen). It has the molecular attributes necessary to interact with the LBC in a manner akin to E2, but it has a higher relative binding affinity (RBA = 178) than E2 (RBA = 100) (Kuiper et al., 1997) and its phenoxy-*N,N*-dimethylethanamine moiety displaces a common helix that forms the boundary between the LBC and AF-2 (see below) so upsetting AF-2 function. This, of course, explains

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Original article

Escherichia coli biotransformation of daidzein fermentation products from soy-based foods—relevance to food oestrogenicity-based functionality

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Summary Oestrogenic isoflavones (e.g. daidzein) present in soy-based foods are likely to be important in food functionality. Biotransformation of daidzein during fermentation-based soy-containing food manufacture forms three trihydroxy metabolites, 6-*ortho*-hydroxydaidzein (6-OHD), 8-*ortho*-hydroxydaidzein (8-OHD) and 3′-*ortho*-hydroxydaidzein (3′-OHD) which alters the oestrogenicity of the final food product. We report that *Escherichia coli* (a key component of the gut microbiome) metabolism converts 8-OHD to 6-OHD with a likely concomitant increase in oestrogenicity. This means that the functionality of 8-OHD-containing soy-based foods is altered by gut microbiome metabolism. This change in oestrogenicity-based food functionality might have benefits for postmenopausal women, while being a feminising health risk for males and could add to the risk of oestrogen-mediated precocious puberty in girls.

Keywords 17β-Oestradiol, daidzein, food functionality, gut microbiome, health risks/benefits, isoflavones, oestrogen mimics, xenoestrogens.

Introduction

A recent World Health Organisation report assessed the State of the Science of Endocrine Disrupting Chemicals (EDCs) and suggested that there are in excess of 800 known EDCs and potentially many more (WHO/UNEP, 2013). A large proportion of these are oestrogen mimics, and many occur as natural components of food (e.g. genistein and daidzein in soy-based foods), either as endogenous plant metabolites (e.g. phytoestrogens) or as feed-derived xenoestrogens in food-producing animals (e.g. phytoestrogens in cows' milk (Höjer *et al.*, 2012)). Oestrogen mimics in food form part of the complex cocktail that humans are exposed to (Shaw, 2014), and are thought to have significant biological effects at a population level (e.g. reduced sperm quality (Geisler *et al.*, 2002)). Conversely, they might have important implications in food functionality; for example, bread rich in seeds (e.g. linseed) containing phytoestrogens is consumed by some menopausal women to ameliorate 17β-oestradiol withdrawal effects.

17β-Oestradiol mimics fall into two main categories, natural and man-made; those foreign to a particular system (e.g. humans) are termed xenoestrogens (from the Greek Xenos meaning foreign). The first synthetic xenoestrogen was diethylstilbestrol (Dodds *et al.*, 1938) and was misguidedly used in pregnancy to reduce the risk of complications and as a growth promoting agent in agriculture. Other examples are bisphenol-A (BPA), a monomer used in the manufacture of widely used polycarbonate plastics; 4-nonylphenol, a non-ionic detergent and plasticiser; and ethinyl oestradiol a key component of the contraceptive pill (Krishnan *et al.*, 1993). Such compounds are key components of the human oestrogen mimic food and drink exposure cocktail; for example, BPA-based plastics and lacquers are used in food packaging, 4-nonylphenol (and other nonylphenols) was used in dishwashing formulae, and ethinyl oestradiol is a drinking water contaminant.

Phytoestrogens include flavones and isoflavones (Fig. 1); isoflavones are commonly found in legumes, such as soybeans. Soybean per se (e.g. in tofu) and soybean products (e.g. soybean meal) are used extensively in food production (e.g. soybean meal is used as an improver in bread manufacture). The extent of soybean use is exemplified by the world soy production in 2016 being estimated to be 326 million tones (USDA, 2016). This multifarious array of oestrogen mimic

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